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Research Papers

Research Notes

Reduction of Proteolytic Activity of the Milk Clotting Enzyme from *Rhizopus oligosporus*

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The milk clotting and proteolytic activities of the crude enzyme preparation of *Rhizopus oligosporus* No. 1104 were compared with some of the commercially used coagulants for cheese manufacture. The proteolytic activity of the enzyme preparation was comparatively higher than those of other coagulants. A considerable reduction of the proteolytic activity in the enzyme preparation was achieved by aluminium trioxide adsorption followed by heat treatment. Fractionation of the enzyme preparation revealed three distinct peaks having different ratios of milk clotting to proteolytic activities. The pooled fractions of the last peak showed five times milk clotting than other fractions.

Cheddar cheese made with heat treated, Al_2O_3 adsorbed and fractionated enzyme preparations had better flavour and texture as compared with cheese prepared with untreated enzyme preparation.

Calf-rennet from the abomasum of suckling calves is the traditional coagulant used in cheese manufacture. In recent years, due to short supply and higher prices of calf-rennet, active search has been made to find out suitable rennet substitutes[1]. Among plant, animal and microbial proteases, fungal coagulants have found greater acceptance as a substitute of calf-rennet for cheese making. In United States alone, nearly 60 per cent of the demand for calf-rennet is met by the proteases derived from *Endothia parasitica*, *Mucor pusillus* and *Mucor miehei*[2].

As most of the rennet substitutes are highly proteolytic, the important factor determining the suitability of enzyme preparation for cheese making, is the ratio of proteolytic activity to milk clotting activity. The high proteolytic activity of a coagulant results in excessive digestion of curd and less retention of fat in cheese[3]. It has also got undesirable effects on the body and flavour of the ripened cheese due to excessive proteolysis during ripening.[4] A strain of *Rhizopus oligosporus* of this laboratory produced an extracellular protease having good milk clotting and proteolytic activities. Cheddar-cheese prepared with this enzyme was organoleptically acceptable but there were slight reductions in the yield of cheese and some defects in flavour and texture[5]. Apparently, the acid-protease of *R. oligosporus* differs from calf-rennet in its high proteolytic activity. With a view to bring it very close to calf-rennet in its activities, attempts were made to adjust the ratio of milk clotting and proteolytic activities. Results of these studies are presented in this communication.

Materials and Methods

Meito rennet (*Mucor pusillus*), Sure curd (*Endothia parasitica*), prozyme (*Streptomyces* sp.) and calf-rennet used in the investigations were supplied by Meito Sangyo Co. Ltd., Japan; Chas. Pfizer & Co. Inc., New York, U.S.A.; Tokyo, Kyowa Hakko Kogto Co. Ltd., Japan and Hansens' Laboratory Denmark, respectively.

Culture and the enzyme: The enzyme taken up for this study was produced by surface fermentation of the mould strain *Rhizopus oligosporus* No. 1104 of the culture collection CFTRI, cultivated on wheat-bran. Water-extract of the mouldy-bran served as source of the enzyme.

Enzyme assay: Milk-clotting activity was determined by the method of Arima[6] and expressed in terms of Soxhlet units. One Soxhlet unit is defined as the amount of enzyme which clots 1 ml of milk (10 per cent (w/w) skim milk powder containing 0.01 M calcium chloride) in 40 min at 35°C. Proteolytic activity at pH 6.0 was measured by the casein digestion method described by Kunitz[7]. The amounts of solubilized protein were determined by the use of Folin-Ciocalteau reagent according to the method of Lowry *et al.*[8] A blank was always taken along with the samples. The proteolytic activity was expressed in terms of μg of tyrosine liberated per ml of enzyme in 20 minutes reaction time.

Adsorption of enzyme: For adsorption studies, 0.5 g of adsorbent was mixed with 5 ml enzyme preparation at pH 4.5 and shaken on a rotary shaker for 10 min.

The mixture was centrifuged at 2500× G and milk clotting and proteolytic activities were determined in the supernatant.

Adsorbents: Fuller's earth, hyfluosupercel, kaolin, kiesselghur, activated charcoal, bentonite, aluminium trioxide, starch and sodium and calcium silicates were used as adsorbents.

Preparation of calcium phosphate gel: The method of Tsubi and Hudson[9] was followed for the preparation of calcium phosphate gel and separation of proteolytic component from the milk clotting fraction of the enzyme preparation.

Preparation of γ alumina gel: The procedure used for γ alumina gel preparation was that of Dixon and Webb[10].

Selective precipitation of crude enzyme preparation: The precipitation of enzyme was carried out at 4°C using different levels of saturation of ammonium sulphate, acetone, ethanol, isopropanol, methanol and combination of isopropanol and methanol. The precipitates obtained after decantation of the supernatant were dissolved in water and tested for milk clotting and proteolytic activities.

Separation by ion-exchange chromatography: The carboxylic ion-exchange resin Amberlite IRC-50 was used in the column. The methods followed for column preparation, equilibration of resin and elution of protein were according to the method of Iwasaki *et al*[11].

Results and Discussion

Data regarding milk clotting and proteolytic activities of various milk clotting enzymes used commercially in cheese manufacture are given in Table 1. It is evident from the results that the enzyme preparation of *Rhizopus oligosporus* had very high proteolytic activity in comparison with that of calf-rennet. The commercially used calf-rennet substitutes of microbial origin such as Meito rennet of *Mucor pusillus* Lindt. and Sure-curd of *Endothia parasitica*, exhibited considerably higher ratio of milk clotting to proteolytic activity when compared with the preparation of *Rhizopus oligosporus*. The highest ratio of milk clotting activity to proteolytic activity was seen in the case of calf-rennet.

Table 1. MILK CLOTTING AND PROTEOLYTIC ACTIVITIES OF VARIOUS COMMERCIAL RENNET PREPARATIONS OF ANIMAL AND MICROBIAL ORIGINS

Rennet preparation	Milk clotting units (Soxhlet units/ml) (1)	Proteolytic activity units (μ / ml Tyrosine) (2)	Ratio of 1 and 29/t
Meito rennet*	2,26,400	32,000	7.07
Prozyme*	68,500	88,000	0.77
Sure curd*	83,150	28,000	2.96
Calf rennet**	21,320	1,000	21,32
*Rhizopus oligosporus***	6,000	4,000	1.5

*Milk clotting activity and proteolytic activity/g.
**Milk clotting activity and proteolytic activity/ml.

A number of procedures were tried in this study to alter the ratio of proteolytic activity to milk clotting activity of the crude enzyme preparation. These included selective adsorption by adsorbents, selective inactivation by heat, selective fractionation and precipitation by salt and solvents and separation by the ion-exchange column chromatography.

Results summarized in Table 2 reveal that among the adsorbents used, only aluminium trioxide and bentonite showed maximum adsorptions of proteolytic activity of the enzyme, leaving most of the milk clotting activity

Table 2. SELECTIVE ADSORPTION OF MILK CLOTTING AND PROTEOLYTIC COMPONENTS OF THE ENZYME PREPARATION OF *Rhizopus oligosporus*

Adsorbents	Milk clotting units (Soxhlet unit/ml)	% recovery of milk clotting units (filtrate)	Proteolytic activity units (μg tyrosine/ml)	% recovery of proteolytic units (filtrate)
Control	8,000	100.0	4,400	100.0
Fuller's earth	7,750	96.8	3,800	86.3
Hyfluosupercel	7,750	96.8	3,800	86.3
Kaolin	8,000	100.0	3.800	86.3
Kiesselghur	7,750	96.8	4,400	100.0
Activated charcoal	6,150	76.8	4,000	90.9
Bentonite	6,000	75.0	2,000	45.4
Aluminium trioxide	8,000	100.0	2,400	54.5
Sodium silicate	3,750	46.8	2,800	63.6
Calcium silicate	6,300	78.7	3,400	77.2

TABLE 3. INFLUENCE OF TEMPERATURE ON THE PROTEOLYTIC AND MILK CLOTTING ACTIVITIES OF THE PROTEASE

Temp* °C	Milk-clotting activity (Soxhlet units/ml)	Proteolytic activity units (μg tyrosine/ml)
RT	5,909	3,000
30	5,912	3,000
40	5,775	2,800
50	5,700	2,200
60	5,500	2,200
70	4,400	300
80	Nil	Nil
90	,,	,,

*Duration of heating: 15 min; RT = Room temperature.

TABLE 4. EFFECT OF HEAT AND ALUMINIUM TRIOXIDE TREATMENT ON PROTEOLYTIC AND MILK CLOTTING ACTIVITIES OF THE PROTEASE

Treatment	Milk clotting activity (soxhlet units/ml)	% loss of milk clotting activity	Proteolytic activity (μg tyrosine/ml)	% loss of proteolytic activity
Control	6,150	—	2,500	—
Heat*	5,000	19	750	70
Al_2O_3	5,850	4.9	1,000	60
Heat* + Al_2O_3	5,000	18.7	375	85
Al_2O_3 + Heat*	4,900	20.4	375	85

*At 62°C for 15 min.

in the filtrate. From practical and economic viewpoints, poor adsorption of milk clotting fraction of enzyme is highly useful as it does not require elution of the protein by different types of buffers from the adsorbents.[12] Silicates which have been reported to be ideal adsorbents[13] for the elimination of non-specific proteolytic enzymes of microbial rennets, were not useful in this study.

Since aluminium trioxide showed maximum adsorption of proteolytic rich fraction without affecting much of the milk clotting activity, variables influencing adsorption by aluminium trioxide, were standardized. Twenty per cent aluminium trioxide at pH 4.5, agitated for 20 min on a rotary shaker was found optimum to adsorb proteolytic component preferentially, thus leaving the milk clotting fraction in the supernatant. Repeated adsorption by aluminium trioxide was also carried out but it did not show any improvement in further reduction of proteolytic activity.

The effect of heating on the selective inactivation of milk-clotting and proteolytic components was studied and results are given in Table 3. The results show that heating the enzyme preparation for 15 min at 62-64°C resulted in nearly 70 per cent reduction in the proteolytic activity. Adsorption with aluminium trioxide in conjunction with heat treatment (Table 4) at 62°C for 15 min resulted in about 85 per cent elimination of proteolytic fraction and retention of 80 per cent milk clotting activity. It may be pointed out that adsorption combined with heat treatment is a simple and feasible approach for the reduction of proteolytic activity of the enzyme preparation.

The most economical and successful methods such as calcium phosphate[14-16] and alumina γ gel[17] and casein[18] used for fractional adsorption of enzymes were not effective in the separation of either milk clotting or proteolytic components of the enzyme preparation. Also, pH adjustment at different ranges was not found useful for separating the proteolytic fraction as reported in many enzyme purifications.[19] At high and low pH ranges, both milk clotting and proteolytic activities were found to be lost completely.

The reduction of proteolytic activity was also attempted by preparation with acetone, ethanol, n-propanol, methanol and ammonium sulphate of different concentrations. The results obtained in this study indicated that fractional precipitation with solvents and salt could not separate out the two components, i.e., milk clotting and proteolytic, substantially. Acetone followed by ethanol and ammonium sulphate, proved to be most suitable precipitating agents, recovering about 76.0, 75.0 and 75.0 per cent respectively of milk-clotting activity of the enzyme preparation. Tannic acid and its salts in different concentrations were also used as the selective precipitants but the overall recovery of protein was extremely poor. A black, gummy and sticky precipitate was obtained with these precipitants. Methanol and its combination with isopropanol in the ratio of 40:30 yielded powdery and white precipitate. The combination of salts and solvents as co-precipitants could not influence much of the separation of milk clotting component from proteolytic component of the enzyme preparation.

It is evident from the data discussed above that the proteolytic activity could not be reduced beyond certain limits either by adsorption or heat-treatment. Therefore, separation by ion-exchange column-chromatography was attempted. A summary of the results of a fractionation procedure is given in Table 5. Amberlite IRC-50 effected good separation. Three well separated peaks were distinguished having different proportions of proteolytic and milk clotting activities. The maximum

TABLE 5. PARTIAL PURIFICATION OF PROTEASE FROM *Rhizopus oligosporus*

Step	Milk clotting activity (Soxhlet) Units/ml (1)	Proteolytic activity (μg tyrosine/ml) (2)	Ratio of 1 and 2	Specific* activity
Enzyme conc.	4,600	3,656	1,26	71.8
Acetone Ppt.	3,800	2,400	1.58	76.0
Amberlite IRC-50				
(*a*) Peak I fractions	35	27.5	1.27	7.2
(*b*) Peak II fractions	198.4	37.4	5.30	793.6
(*c*) Peak III	305.28	46.62	6.54	1526.4

*Specific activity: Milk clotting activity/mg of protein.

proteolytic and minimum milk clotting activities were observed in fractions of first peak, eluted by 0.1-0 2 M (pH 3.5) acetate buffer, while the fractions of the second peak contained relatively less proteolytic activity. The fractions of third peak eluted by 0.2-0.5 M acetate buffer at pH 5.0 accounted for highest milk-clotting activity, with limited proteolysis. The last peak also showed a substantial degree of purification as evidenced by 21-fold increase in the specific activity and 5-fold increase in the proteolytic to milk clotting ratio. Although Tauber and Laufer,[20] and Krishnamurti and Subrahmanyan[12] separated the proteolytic fraction from milk clotting fraction of some plant proteases, it was not possible in this study to separate them. However, as has been observed in our study, multiple components having different ratio of milk clotting to proteolytic activity were found in the enzyme preparations of *Physarum polycephalum*[21], *Rhizopus oligosporus* NRRL-327[122] and *Mucor miehei*[23] Alais and Novak[24] reported three fractions with different percentages of proteolytic activity in the enzyme preparation of *Endothia parasitica*, but they could not separate out highly active milk clotting activity from proteolytic activity.

Heated aluminium trioxide adsorbed and precipitated and fractionated enzyme preparations (fractions of peak No. 3) were used for cheese making. Cheddar cheese prepared with treated enzyme had better flavour and texture than the one prepared with crude enzyme. Further work on the chemical composition of the above experimental cheese during ripening is in progress. Physical and chemical properties of the enzyme proteins of three different peaks will be reported separately.

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Studies on Refining and Storage of Rice Bran Oil*

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Refining of rice bran oil from parboiled paddy before and after dewaxing was studied in laboratory and pilot plant experiments. The refining losses varied from 20.0 to 34.0 percent for the oil containing wax and from 14.0 to 19.0 percent for the dewaxed oils. Incorporation of molasses prior to alkali refining reduces the refining losses from about 20 to 14 per cent, representing a 30 per cent reduction. Storage of raw, refined and bleached rice bran oils for 370 days at ambient temperature showed a slight and gradual rise in free fatty acid content, a fair rise in peroxide value especially in neutralised and bleached oils, a slight decrease in colour of crude and bleached oils, and considerable bleaching of neutralized oils.

In an earlier paper,[1] refining of experimental rice bran oil (RBO) extracted from raw rice bran was studied. Currently about 80,000 tonnes of RBO are being produced, of which only 5,000 tonnes are said to be of edible grade. This rise in production has occurred because RBO has been permitted as one of the constituent oils for the manufacture of *vanaspati*. With general increase in the demand for edible oils and the forthcoming statuatory permission for the use of refined RBO directly as a cooking oil, greater demand will occur for edible RBO.

Work was undertaken in collaboration with the Paddy Processing Research Centre, Thiruvarur, Tamil Nadu, to study the refinability of RBO. The shelf life of edible oils is important to their economy and nutrition. Large quantities of RBO of industrial quality are being currently produced. Now that problems of stabilization of rice bran, and dewaxing and refining of its oil are being solved, and since statuatory recognition for rice bran oil is imminent, storage of edible grade oil assumes significance. A comparative study of the keeping qualities of raw, neutralized and bleached RBO has, therefore, also been made.

Materials

Solvent extracted rice bran oil obtained from heat-stabilized rice bran of parboiled paddy was used in this study. Molasses was procured from a sugar factory. Jaggery was of market sample.

Methods

Refining on a laboratory scale was done according to the AOCS Official Method recommended for peanut oil. On a pilot plant scale, about 8 kg of RBO was neutralized in the cold with alkali lye of predetermined strength and excess for 30 min. at the end of which the mass was heated to 65°C. The mixture was then passed through a Delaval centrifugal separator to get neutralized oil. Soapstock remained in the bowl. Additive, where used, was added as a 15 percent solution either along with or prior to alkali lye. Bleaching of neutralized oil was carried out following AOCS Official Method using a combination of 3 percent earth (Tonsil AC) and 0.3 percent carbon (Darco). Raw, neutralized and bleached oils were stored in clean air tight tin containers (5 kg capacity) at ambient temperature for more than a year. Samples were drawn at 30-day intervals and analysed for free fatty acid content, iodine value and peroxide value according to AOCS methods. Lovibond Tintometer colour was read in a 2.54 cm cell. Results are given in Tables 1, 2 and 3.

Results and Discussion

Refining: From Table 1, it is seen that refining losses of RBO are rather higher than one would expect for an oil of 3.9 free fatty acid content. The presence of monoglycerides and other hydroxylated compounds (emulsifying agents) may be responsible[2]. The refining losses vary from 20 to 34 percent, the refining factors (obtained by dividing refining loss by FFA) being 5.1 to 8.7. Use of alkali lyes between 12° to 20° Baume in strength give the same range of losses. It is the excess of alkali that seems to influence the refining loss, the highest loss occurring with a maximum of 20° Be' alkali.

Dewaxing of oil before refining has beneficial effect in reducing the refining losses as seen from Table 2. Dewaxing the oil by centrifuging without prechilling yielded 4-6 percent of crude wax sludge. Leaching this

*This is the 19th paper on rice bran and rice bran oil published by the Institute

TABLE 1. REFINABILITY OF UNDEWAXED RICE BRAN OIL FROM BRAN MILLED FROM PARBOILED RICE

Alkali lye used	Scale of experiment	Refining loss %	Refining factor*	Lovibond colour in 2.54 cm cell					
				Neutralised oil			Neutralized and bleached oil		
				Y	R	B	Y	R	B
12° Be', max	Centrifugal pilot plant	22.5	5.7	25	2.4 (37.0)	0.5	9.5	0.9 (14.0)	0
16° Be', 80% excess	AOCS Cup	21.0	5.4	32	4.5 (54.5)	1.1	10.5	1.4 (17.5)	0
20° Be', 80% excess	,,	21.7	5.6	27	3.9 (46.5)	0.5	9.0	1.5 (16.5)	0
20° Be', 80% excess	Centrifugal pilot plant	20.0	5.1	25	4.6 (48.0)	0.0	16.5	2.8 (30.5)	0
20° Be', max Citric acid-treated	,,	34.0	8.7	20	3.2 (36.0)	0.2	8.5	1.1 (14.0)	0

Lovibond colour of the original oil = 35Y + 8.9R + 2.5B, visual colour, dirty brown
Figures in parenthesis are Y + 5R units
FFA of the crude oil is 3.9%

$$\text{*Refining factor} = \frac{\text{Refining loss}}{\text{FFA}}$$

TABLE 2. REFINABILITY OF DEWAXED RICE BRAN OIL FROM BRAN MILLED FROM PARABOILED RICE

Alkali lye used	size of experimenta unit	Refining loos %	Refining factor	Lovibond colour in 2.54 cm cell					
				Neutralized oil			Neutralized and bleached oil		
				Y	R	B	Y	R	B
12° Be', max	Centrifugal pilot plant	19.0	4.8	24	2.8 (38.0)	0.6	10.0	1.0 (15.0)	0
20° Be', 80% excess	AOCS Cup	16.0	4.1	16	4.7 (39.5)	0.6	10.5	1.6 (18.5)	0
20° Be', 80% excess	Centrifugal pilot plant	16.5	4.2	23	2.1 (33.5)	1.3	11.0	1.4 (18.0)	0
20° Be', 80% excess High speed (5500 rpm) 2 min stirring	,,	18.5	4.7	22	3.3 (38.2)	0.7	11.0	1.2 (17.0)	0
20° Be', 80% excess sod. silicate additive	,,	18.7	4.8	21	1.5 (28.5)	0.1	10.0	1.1 (15.5)	0
20° Be', 80% excess	,,	17.4	4.4	22	2.8 (36.0)	0.4	11.0	1.4 (18.0)	0
20° Be', 80% excess molasses additive	,,	14.0	3.6	22	1.7 (30.5)	0.5	9.2	2.0 (19.2)	0

Lovibond colour of the dewaxed oil = 35Y + 8.3R + 2.7B, visual colour, reddish brown
Figures in the parenthesis are Y + 5R units

$$\text{Refining factor} = \frac{\text{Refining loss}}{\text{FFA}}$$

TABLE 3. STORAGE BEHAVIOUR OF CRUDE (C), NEUTRALIZED (N) AND BLEACHED (B) OILS FROM HEAT-STABILIZED RICE BRAN OF PARBOILED VARIETY

Storage period (days)	Free fatty acid			Iodine value (Wijs)			Peroxide value*			Lovibond colour in 2.54 cm cell — Y+5R units (Integer)		
	C	N	B	C	N	B	C	N	B	C	N	B
Initial	4.5	0.18	0.22	95	95	96	15	13	5	80	42	17
30	—	0.24	0.29	—	95	94	—	40	24	—	—	21
60	4.7	0.24	0.28	93	94	95	16	23	24	78	31	16
90	4.7	0.26	0.30	92	93	93	20	32	28	77	30	15
120	4.8	0.26	0.31	91	93	92	20	37	36	77	30	15
180	4.9	0.27	0.36	—	—	—	—	—	—	76	29	16
240	5.2	0.27	0.37	90	92	92	26	45	53	75	29	16
300	5.7	0.28	0.40	90	92	—	26	56	60	75	26	—
370	5.9	0.40	0.44	92	93	94	30	63	72	72	24	15

*Peroxide value = meq of peroxide oxygen/kg of fat

crude repeatedly with cold acetone gave an yield of 1.4 per cent true wax. The refining losses vary from 14 to 19 per cent (refining factors 3.6 to 4.8) which are consistently lower than the corresponding values for undewaxed oil. Addition of molasses or jaggery reduces the refining losses. The refining loss of RBO when 2 percent molasses was used as an additive during neutralization is the lowest recorded, (14 percent). In separate refining studies on RBO derived from raw paddy, the refining loss of the oil (free fatty acid content 10 per cent) was 50 per cent without and 26 per cent with the use of jaggery as additive. The reason for this is not yet known.

The colours of the neutralized and bleached oils in all cases conform to the Indian Standard Specification (IS:3448-1968) requirements for refined rice bran oil, namely, colour in 1 in. cell, Y+5R units=20 with no dominant green colour.

The soapstock of RBO is loose and settles very slowly, and entrains much oil resulting in higher refining losses. Batch refining and gravity settling of soap as for other oils would be unsuitable. A rapid neutralization step followed by centrifugal separation of soapstock would be a more satisfactory way of bringing increasing quantities of RBO with free fatty acid contents of even as high as 10 per cent into the edible fold.

Keeping quality: Table 3 shows the keepability of RBO.

Free fatty acid content: The free fatty acid content of raw oil rose from 4.5 to 5.9, of neutralized oil from 0.18 to 0.40 and of bleached oil from 0.22 to 0.44 in 370 days.

Iodine value: The values fluctuate erratically over a narrow range through experimental vagaries, and storage has no significant effect on the iodine value of raw as well as processed RBO.

Peroxide value: Storage has considerable effect on peroxide values. In crude oil, the value rose in one year from 15 to 30. In neutralized oil, the value rose from 13 to 62 and in bleached oil from 5 to 72.

Lovibond Tintometer colour: The colours of raw oils were reduced slightly and gradually from 80 units to 72. There was no diminution in the yellow colour but only in the red colour. Colours of bleached oils remained more or less constant while the colours of alkali-neutralized oils registered noticeable reduction from 42 to 24.

Acknowledgement

Our thanks are due to Dr. V. Subrahmanyan, Head, Paddy Processing Research Centre, Thiruvarur, Tamil Nadu who has been an inspiration behind these studies. Our thanks are due to Modern Rice Mill and Solvent Extraction Plant (Food Corporation of India), Sembanarkoil, Tamil Nadu, for supplying rice bran oil. The work was done under a scheme financed by the Indian Council of Agricultural Research, New Delhi to whom our thanks are due.

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Lipid Profile and Fatty Acid Composition of Finger Millet (*Eleusine coracana*)

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Total lipids constituting 1.85-2.10% from seven breeding varieties of finger millet (ragi, *Eleusine coracana*) were extracted with chloroform-methanol, purified, resolved by silicic acid column chromatography and analysed for component fatty acids. The lipid consists of 70-72% neutral lipids, mainly triglycerides and small proportion of sterols, 10-12% of glycolipids and 5-6% of phospholipids. Proportions of lipid classes were similar in all seven ragi varieties. Chloroform-insoluble lipids constituted 8-10%. All classes of lipids contain 46-62% oleic acid, 8-27% linoleic acid, 20-35% palmitic acid, and traces of linolenic acid. Cultivars within a species exhibited identical lipid profiles.

Finger millet (*Eleusine coracana*) is grown in various regions of India particularly in Karnataka and southern Maharashtra, where it is consumed in large quantities by low socio-economic groups of people because of its comparatively low price. It has 7 per cent protein, exceptionally high calcium (344 mg per cent) and substantial iron (17.4 mg per cent).[1] Since the poorer sections of Indian society cannot afford to buy the normal requirements of cooking oil, the nature of the lipids in finger millet could be of some nutritional significance in areas in which it is consumed.

The lipid and fatty acid composition of millets like barley,[2-5] bajra or pearl millet,[6] Italian millet[7] and oats[8-10] have been studied. The present paper describes the different classes of lipids and component fatty acids from seven breeding varieties of finger millet.

Materials and Methods

Seven cultivars of finger millet, viz, 'PR 202', 'HPB 7-6', 'Indaf I', 'ECW 1360', 'EC 4840', 'Hamsa' and 'HES 929', were obtained from the University of Agricultural Sciences, Hebbal. The first five were brown in colour and the last two white. Reagents and solvents used were of analytical grade.

The seeds were finely ground in a mill, dried at 50°C to constant weight and 25 g portions were Soxhlet-extracted with chloroform-methanol (2:1 v/v, 8-10 hr) using tocopherol acetate in chloroform as an antioxidant. Following solvent removal in a rotary flash evaporator under nitrogen, the lipids were stored in chloroform-methanol at –20°C. Non-lipid contaminants were removed by passing through Sephadex G-25 (100-300 μ, Sigma, USA)[11] and lipids recovered from the eluates weighed. When redissolved at once in chloroform, a portion of this lipid did not go into solution. This portion, designated chloroform-insoluble lipid, was examined separately. The chloroform-soluble fraction was resolved on a 100-200 mesh silicic acid column (V. P. Chest Inst., New Delhi) employing chloroform (neutral lipids), acetone (glycolipids) and methanol (phospholipids).[12] The eluates were concentrated in a rotary flash evaporator under nitrogen and weighed. Total recoveries amounted to 85-90 per cent. An aliquot of the neutral lipid fraction was used to estimate total sterols; free sterols were determined by digitonin precipitation followed by estimation as for total sterols and esterified sterols by difference.[13,14]

Methyl esters were derived from total lipids and neutral lipids by transmethylation with 14 per cent boron trifluoride in methanol, followed by extraction with n-heptane and concentration under a stream of nitrogen.[15] Glycolipids, phospholipids and chloroform-insoluble lipids were transmethylated using 3 per cent methanolic HCl for 2 hr.[16] For gas chromatography, a

Table 1. Total lipids, chloroform-soluble and chloroform-insoluble lipids (% wt.) of different varieties of finger millet

Varieties	Total lipids	Chloroform-soluble lipids	Chloroform-insoluble lipids
PR 202	2.10	1.90	0.20
HPB 7-6	2.00	1.83	0.17
ECW 1360	1.95	1.78	0.17
EC 4840	2.05	1.86	0.19
INDAF I	1.85	1.70	0.15
HAMSA	2.00	1.84	0.16
HES 929	1.95	1.70	0.25

TABLE 2. DIFFERENT LIPID CLASSES OF FINGER MILLET VARIETIES (% WEIGHT)

Lipid classes	PR 202	HPB 7-6	ECW 1360	EC 4840	INDAF I	HAMSA	HES 929
Neutral lipids	1.46	1.38	1.35	1.44	1.30	1.40	1.38
Glycolipids	0.25	0.26	0.23	0.27	0.25	0.26	0.24
Phospholipids	0.12	0.10	0.11	0.12	0.10	0.11	0.10
Neutral lipid composition							
Glycerides, FFA and hydrocarbons	92.00	93.00	92.00	92.80	93.40	93.00	92.40
Sterols	7.20	6.20	7.10	6.30	5.90	6.40	6.60
Sterol esters	0.80	0.80	0.90	0.90	0.70	0.60	1.00

Varian Aerograph 1400 series with a flame ionization detector was used (column 8 ft × 1/8 in. 15 per cent DEGS on Chromosorb W, 185°C). Unknown peaks were identified through standards. Unsaturated fatty acid methyl esters were confirmed by bromination and subsequent disappearance on gas chromotograms. Peak areas were determined by triangulation, no correction factors being used.

Results and Discussion

Table 1 gives the percentage (average of three determinations) of total lipids, chloroform-soluble lipids and chloroform-insoluble lipids in the seven varieties of finger millet. Total lipids constituted nearly 2 per cent in all the ragi cultivars. Chloroform-insoluble lipids constituted 8-10 per cent of the total lipid extracted initially using chloroform-methanol. This fraction

TABLE 3. FATTY ACID COMPOSITION (% WEIGHT) OF DIFFERENT CLASSES OF LIPIDS OF FINGER MILLET

		PR 202	HPB 7-6	ECW 1360	EC 4840	INDAF I	HAMSA	HES 929
Neutral lipids	16:0	25.0	25.6	26.0	25.0	25.3	25.2	24.0
	18:0	Tr	—	—	—	—	—	—
	18:1	49.4	49.0	48.0	49.0	49.2	49.0	50.3
	18:2	24.8	24.6	25.0	25.0	24.7	25.0	25.1
	18:3	0.8	0.8	1.0	1.0	0.8	0.8	0.6
Glycoplipids	16:0	30.0	31.9	30.0	32.0	32.0	24.6	26.7
	18:0	Tr	Tr	Tr	Tr	Tr	Tr	Tr
	18:1	61.5	61.3	62.1	61.0	61.7	63.9	62.4
	18:2	8.5	6.8	7.9	7.0	6.3	11.5	10.9
	18:3	Tr	Tr	Tr	Tr	Tr	Tr	Tr
Phospholipids	16:0	25.8	24.3	26.0	25.6	26.6	37.5	34.5
	18:0	Tr	Tr	Tr	Tr	Tr	Tr	Tr
	18:1	46.4	47.9	46.8	48.2	46.0	41.2	43.4
	18:2	26.7	26.4	26.0	25.2	26.3	20.5	21.5
	18:3	1.1	1.4	1.2	1.0	1.1	0.8	0.6
Chloroform-insoluble lipids	16:0	20.0	22.0	20.0	21.5	22.0	21.0	21.0
	18:0	1.5	Tr	2.0	1.0	1.5	Tr	1.5
	18:1	50.5	50.0	49.0	50.0	50.0	51.0	49.5
	18:2	26.0	26.5	27.0	26.0	25.0	26.0	26.0
	18:3	2.0	1.5	2.0	1.5	1.5	2.0	2.0
Total lipids	16:0	25.0	25.6	25.0	26.0	25.0	23.0	23.5
	18:0	Tr	Tr	Tr	Tr	Tr	Tr	Tr
	18:1	49.8	49.0	50.0	48.5	50.0	51.5	50.0
	18:2	24.1	24.3	24.0	24.5	23.2	24.0	25.0
	18:3	1.1	1.1	1.0	1.0	1.8	1.5	1.5

contained only a trace of the lipid phosphorus, and more than 95 per cent of it passed into the chloroform-solubles.

Table 2 shows the proportions (averages of three determinations) of lipid classes in the chloroform-soluble lipids. Neutral lipids are the major group, comprising 70-72 per cent of total lipid (1.3-1.4 per cent of the seed weight), followed by glycolipid 10-12 per cent and phospholipids 5-6 per cent. Neutral lipids consist mostly of triglycerides accompanied by very small proportions of partial glycerides, free fatty acids and hydrocarbons. Total sterols constitute 7-8 per cent of the neutral lipids, and are mostly free sterols accompanied by one per cent of sterol esters.

The proportions of neutral lipids in the present investigation are in agreement with that recorded earlier[1] for lipids extracted with petroleum ether. The phospholipid content of this millet is lower than that of barley in which it constitutes 20 per cent of total lipid. However, proportions of glycolipid and neutral lipid are similar to that of barley, the total lipid content of which ranges from 3.12 to 3.56 per cent in six varieties studied[4].

The chloroform-insoluble fraction appeared to be a saponifiable complex of lipids and proteins, with the former constituting the major portion. The existence of stable complexes of protein and lipids in plant sources has been reported in wheat lipid extracts[17-19].

Table 3 shows fatty acid compositions (average of three determinations) of the total lipids and lipid classes. The fatty acids present in neutral lipids, phospholipids and chloroform-insoluble lipids resemble each other. Palmitic acid ranges from 25 to 30 per cent and is often the only saturated fatty acid in these lipid fractions, infrequently accompanied by a trace of stearic acid. Oleic acid is the predominant unsaturated fatty acid, constituting 61-64 per cent in the glycolipid fraction and 46-51 per cent in other fractions. Linoleic acid content is only 8-12 per cent in the glycolipids, and 23-27 per cent in the other fractions.

The two white varieties of finger millets, 'Hamsa' and 'HES 929', do show some characteristics which appear to distinguish them from the brown varieties. The glycolipids contain distinctly less palmitic acid (24.6 and 26.7 per cent, as against 30-32 per cent), and the phospholipids more palmitic acid (37.5 and 34.5 per cent, as against 24-26 per cent), and generally both oleic and linoleic acid levels are lower in the white varieties. Whether this is fortuitous or otherwise is not clear.

The proportions of saturated and unsaturated fatty acids reported here agree with an earlier report on the fatty acids of millets[5]. The finger millet lipids studied here have notably large quantities of oleic acid (*ca* 50 per cent) and resemble that of oats.[2] The gross lipid composition is quite similar in all the seven cultivars of finger millet. The fatty acid profiles of the constituent lipid classes are also generally similar, except the two white varieties which diverge in certain details from the others.

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Histological and Textural Changes in Muscle Fibre of Mutton During Aging and Freeze Drying

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Histological and textural changes in muscle fibres of fresh mutton during aging and freeze drying were studied. Temperature and duration of aging both affected the textural characteristics and histology of the muscle. Aging for 72 hr at 5°C or 6 hr* at 28°C resulted in better distinction of the fibres with progressive development of kinks, waves, weakening and breakage of the fibres and hence tenderization. Histological pattern varied from muscle to muscle and this affected the tenderness also. The aged muscles disintegrated upon cooking and freeze drying due to cellular destruction. Age of the animal and sex both affected the tenderness. Muscles of male sheep in any age group had higher hardness score than the muscles of the female sheep from the corresponding group.

Texture is one of the most important quality characteristics of meat and is composed of such properties that arise from the structural elements[1]. When applied to meat it means the manner or disposition of union of different types of cells and tissues as perceived by the sensory organs and could be measured by mechanical means in the units of mass and force[2]. Since aging improves the texture and tenderness[3-7] of meat, histological studies were undertaken to study the effect of the time and temperature of conditioning upon muscle fibres in relation to different textural parameters measured objectively, as well as to record the histological changes occurring in the aged mutton fibres during cooking, freezing and freeze drying.

Materials and Methods

Male as well as female sheep of Bannur variety in the three age groups namely, lamb (less than one year old) yearling (one to two and a half years) and mature (3 yr and above) were used in the study. The animals of identical live weight were selected in each group as replicates. They were given water *ad libitum* and slaughtered manually by single stroke method. The carcass was skinned, eviscerated and superficial fat removed. Each carcass was divided longitudinally into two halves. One half was aged and the other half served as control. The dressed carcasses were hung through achilles tendon and aged at 28°C (room temperature) for 6 hr and at 5°C for 72 hr in a walk-in-cooler (RH 90±5 per cent). Longissimus dorsi(LD), Triceps brachii(TB) and Rectus femoris(RF) muscles were selected for histological and textural studies. The samples were removed at 0, 24, 48 and 72 hr of aging at 5°C and at 0, 2, 4, 6, 8 and 24 hr at 28°C. The aged mutton samples were cooked in meat, cooking forms for $4\frac{1}{2}$ hr at an internal temperature of 65°C. The mutton blocks were removed after cooling the forms at 5°C and sliced into $\frac{1}{2}$ in chunks. The sliced mutton was blast frozen in trays to −30°C and freeze dried below surface temperature of 50°C at 0.35 mm of Hg. The dried mutton chunks were packed in tin cans under nitrogen.

For histological studies the muscle samples were fixed in 10 per cent formalin for 40 hr, then washed in running tap water for 8 hr, dehydrated in increasing percentage of alcohol, cleaned in xylene and embedded in paraffin wax blocks containing 1 per cent bees wax. 10 μ thick sections were cut on a rotary microtome, wax removed, sections rehydrated and stained as required. The stains used were Eosin and Hematoxylene for general examination whereas periodic acid and Schiff's stain were used to stain the glycogen. Photo micrographs were taken at 160X. Objective measurement of texture in terms of hardness, cohesiveness, springiness, chewiness, gumminess was done on texturometer,[8] (General Foods Corporation, New York).

Results and Discussion

Histology: Fresh muscle showed either nil or very poorly differentiated muscle fibres (Fig 1A) with interspread spaces due to fat and connective tissue. Upon aging at 5°C, distinction of muscle fibres began after 6 hr and by 24 hr the fibres became well defined and distinct. During this time, fibres develope dkinks and wavy appearance (Fig. 1B). Between 24 and 48 hr of aging, the fibres became weak and breaks started appearing (Fig 1C) and the fibres separated from each other. At the end of 72 hr of aging severe fragmentation of fibres was observed (Fig. 1D). Further aging upto 120

TABLE 1. COMPARISON OF DIFFERENT TEXTURE CHARACTERISTICS AS AFFECTED BY DIFFERENT MUSCLE CLASSES AND BOTH SEX IN BANNUR SHEEP

		Hardness	Cohesiveness	Springiness	Gumminess	Chewiness
Lamb		4.40	0.53	2.96	1.95	7.21
Yearling		5.08	0.69	3.11	3.53	11.60
Mature		7.84	0.61	3.14	4.73	14.85
B. SEX						
(*i*) Within class						
Lamb	M	5.02	0.70	2.90	2.53	10.33
	F	3.78	0.36	3.02	1.09	4.09
Yearling	M	5.30	0.80	3.08	4.18	14.22
	F	4.87	0.59	3.13	2.88	8.99
Mature	M	9.35	0.59	3.13	5.32	17.03
	F	4.99	0.52	3.10	2.77	8.59
(*ii*) Between class						
	M	6.55	0.69	3.04	4.04	13.86
	F	4.99	0.52	3.10	2.77	8.59
C. MUSCLES						
(*i*) Within class						
Lamb	TB	4.98	0.45	3.03	2.29	6.96
	RF	4.15	0.64	2.75	2.67	7.26
	LF	4.08	0.51	3.10	0.89	7.42
Yearling	TB	5.38	0.71	3.85	3.81	13.60
	RF	5.25	0.67	3.13	3.54	11.04
	LD	4.63	0.70	3.15	3.24	10.16
Mature	TB	10.43	0.69	3.10	7.22	22.46
	RF	7.80	0.50	3.13	3.57	11.17
	LD	5.30	0.64	3.20	3.42	10.93
(*ii*) Overall	TB	6.93	0.62	3.06	4.44	14.24
	RF	5.73	0.50	3.00	3.26	9.82
	LD	4.66	0.61	3.15	2.52	9.50

M = Male; F = Female; T.B. = Triceps brachii; RF = Rectus femoris; LD = Longissimus dorsi.

hr, did not affect the histological appearance. The cause of fragmentation of fibres may be Z-line instability and/or catheptic activity of the enzymes of the muscles itself[9-18].

The pattern of histological changes during aging at 28°C was similar to that of aging at 5°C. Because of the elevated temperature changes were very fast. Only 2 hr after aging at 28°C the fibres became distinct; kinks and waves appeared. Breaks in the muscle fibres appeared at the end of 4 hr (Fig. 2A) and became well pronounced after 6 hr of aging (Fig 2B). This stage corresponded to 72 hr of aging at 5°C.

Cooking of the aged mutton further enhanced the degree of breaks in muscle fibres, (Fig 1E) probably due to heat denaturation and shrinking of the fibres. Freezing did not affect the structural integrity, however the fibres were seen more compact. Upon freeze drying, the "band-pattern" of muscle fibres was completely lost and numerous breaks appeared (Fig 1F).

While comparing the effect of aging on LD, TB and RF muscles it was found that the fibres in fresh LD were poorly differentiated, in RF slight differentiation was seen and in TB they were well defined. Histological changes were highly pronounced in TB and least in LD muscle. This kind of differentiation from muscle to muscle has been reported by other workers also[19,20] and seems to be due to constitution of muscle itself, its physiological function, connective tissue content and anatomical position.

Soon after slaughter and storage at 5°C the glycogen content of muscles remained unaffected upto 6 hr (Fig 3A) (prerigor period) subsequently within 24 hr it was reduced to very low or negligible amount (Fig 3B).

Texture: Effect of age and sex on the textural characteristics on selected muscles has been presented in Table 1. It will be seen that with increase in age the hardness of muscle also increased. Similarly in male animals

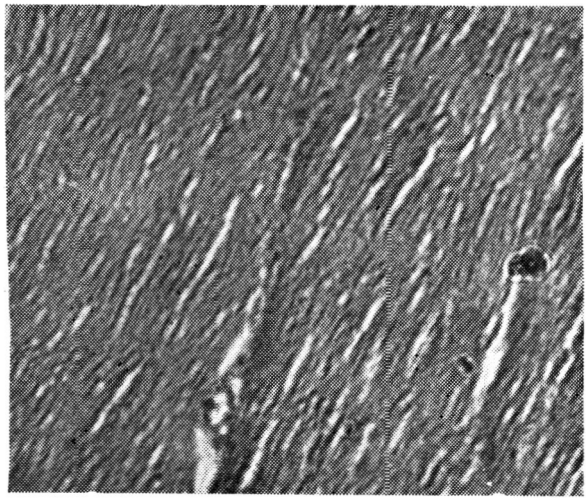

Fig. (1A)—Fresh 0 hr showing poorly differentiated muscle fibres.

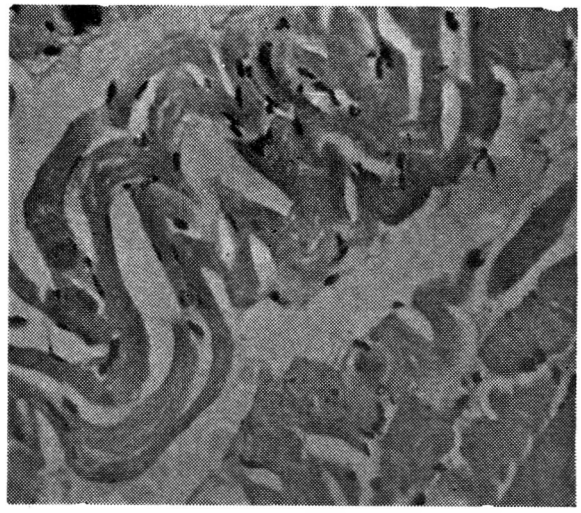

Fig. (1B)—Aged for 24 hr muscle fibres demarcated and defined.

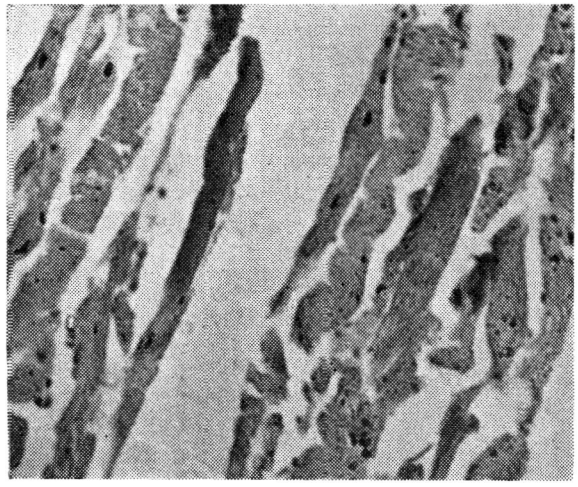

Fig. (1C)—Aged for 48 hr muscle fibres well defined and breaks appeared.

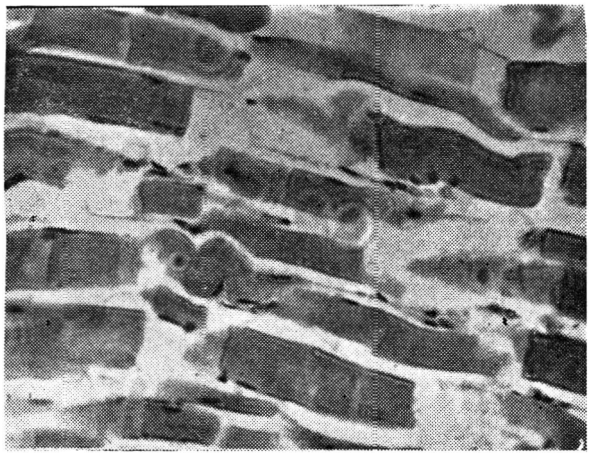

Fig. (1D)—Aged for 72 hr muscle fibres further broken into small strands.

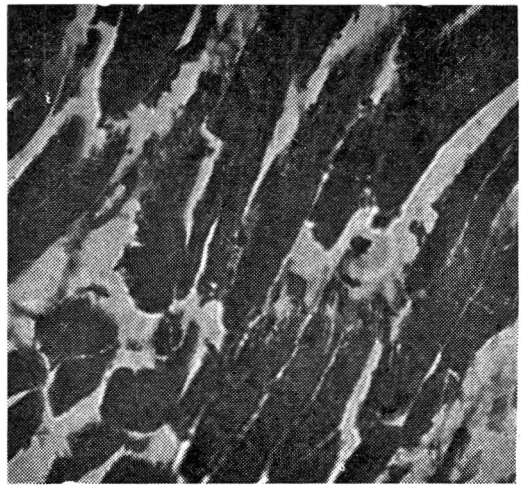

Fig. (1E)—Cooked at 65°C for 4½ hr after aging. Muscle fibres disintegrated and definite breaks.

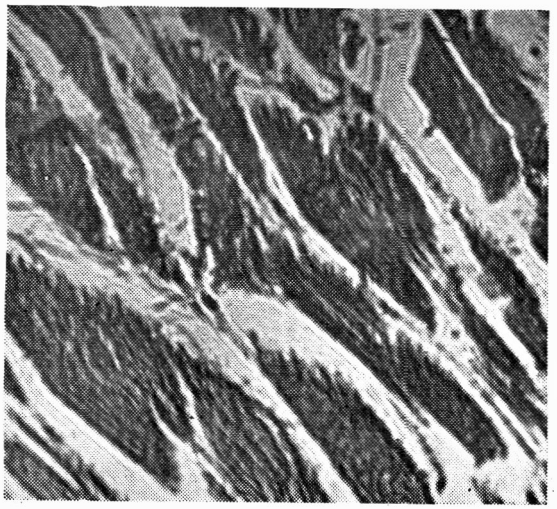

Fig. (1F)—Freeze dried muscle fibre. Disintegrated and no definite pattern maintained.

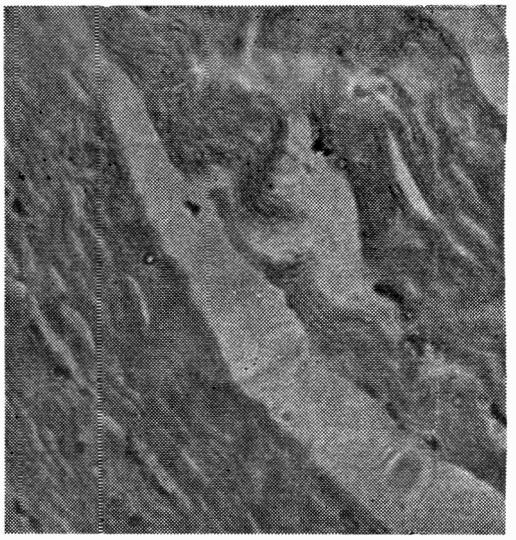

Fig. (2A)—Aged for 4 hr muscle fibres well defined and breaks appeared.

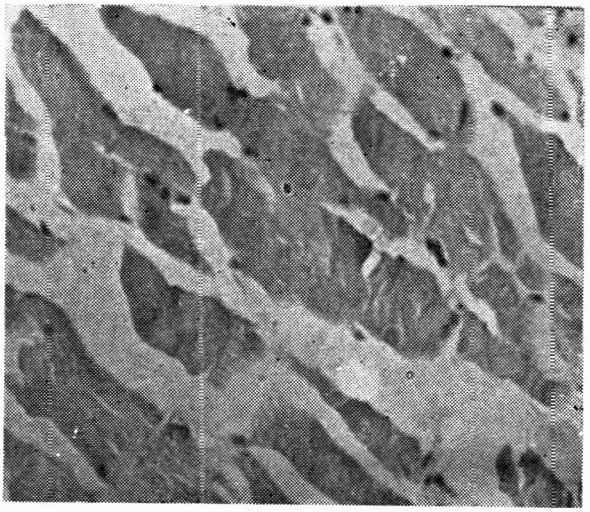

Fig. (2B)—Aged for 6 hr muscle fibres broken into small strands.

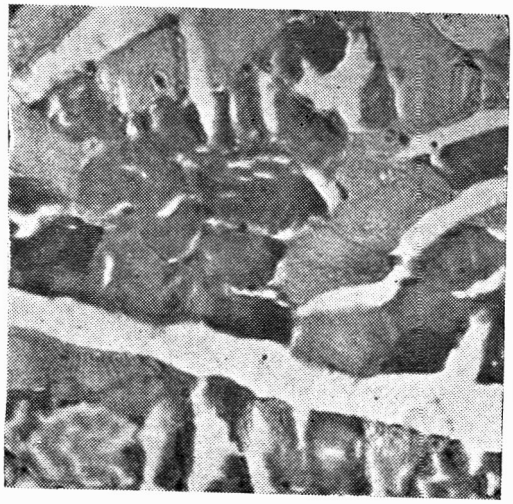

Fig. (3A)—Aged for 6 hr not much of change in glycogen content.

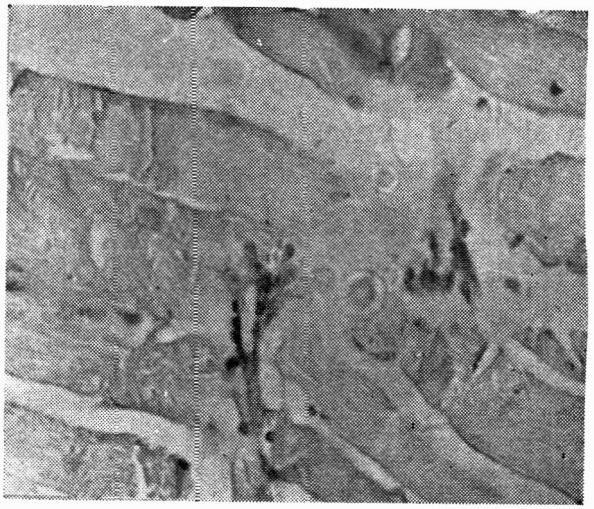

Fig. (3B)—Aged for 24 hr negligible and very low amount of glycogen.

TABLE 2. CHANGES IN TEXTURE PROFILE DURING AGEING* AT 28°C AND 5°C

Time (hr)	(Days)	Hardness 28°C	Hardness 5°C	Cohesiveness 28°C	Cohesiveness 5°C	Springiness 28°C	Springiness 5°C	Chewiness 28°C	Chewiness 5°C	Gumminess 28°C	Gumminess 5°C
0	0	14.44	15.76	0.68	0.75	3.1	3.0	29.52	35.07	9.57	11.55
2		17.80	—	0.66	—	3.1	—	37.81	—	12.11	—
4		17.78	—	0.68	—	3.1	—	39.09	—	12.64	—
6		13.48	—	0.65	—	3.1	—	27.98	—	9.11	—
24	1	11.04	17.57	0.64	0.72	3.1	3.0	22.91	38.36	7.25	12.66
	2	—	17.47	—	0.70	—	3.1	—	38.67	—	12.36
	3	—	16.38	—	0.62	—	3.1	—	31.75	—	10.01
	4	—	16.93	—	0.68	—	3.1	—	35.13	—	11.46
	5	—	17.67	—	0.63	—	3.1	—	34.30	—	11.22
	6	—	18.83	—	0.58	—	3.1	—	34.02	—	10.96
	7	—	15.56	—	0.56	—	3.1	—	27.28	—	8.94
	8	—	15.07	—	0.60	—	3.2	—	28.16	—	8.85
	9	—	12.60	—	0.72	—	3.1	—	28.14	—	9.11
	12	—	11.80	—	0.68	—	3.1	—	24.77	—	8.09

*Leg Rectus Femoris muscle of male yearling (Sheep) carcass voltage 0.25.

irrespective of age or within the age group, the corresponding muscles were harder than in females. Muscle to muscle variation in tenderness was observed. Not much change in cohesiveness and springiness was observed due to variation in age or sex. Gumminess increased with increase in age and the male animals had a comparatively higher value than females. TB had maximum and LD had minimum gumminess value.

Effects of aging at 28°C or at 5°C on the textural characteristics using RF muscle of male sheep have been reflected in Table 2.

During the period of rigor setting that is upto 4 hr at 28°C and 24 hr at 5°C hardness score increased, and then the resolution of rigor started which resulted in tenderization and fall of hardness score. After 6 hr at 28°C and 72 hr at 5°C the mutton became tender. These findings correlate with histological observations. Further aging if continued at either temperature only added to bacterial spoilage and did not help in tenderness improvement. Cohesiveness, chewiness and gumminess values decreased during aging suggesting tenderization. No change in springiness was noticeable. Cooking at 65°C caused slight increase in hardness values due to protein denaturation, shrinkage of muscle fibres and water loss. Freezing did not alter the histological structure of muscle fibres. Neither freezing nor freeze drying affected the textural profile of the cooked mutton.

Acknowledgement

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Immunological Behaviour of Some Major Proteins of Fowl's Egg White in Double Gel Diffusion Patterns

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Native egg albumen and each of its six pure fractions were tested immunologically on Ouchterlony plates by double gel diffusion against their antisera. A minimum number of three antigenic components were found for conalbumin 1, conalbumin 2, ovomucoid and avidin of the native egg albumen whereas its ovalbumin and lysozyme fractions had only two such components. Testing of pure proteins also showed same minimum numbers of antigenic components with their respective antisera.

It is observed that antigenically there is only one type of conalbumin in the egg white although electrophoretically and/or chemically they can be distinguished in two or more types. It is further observed that one antigenic component is common to the major egg proteins except for lysozyme which apparently is much more specific than others.

The immunoelectrophoresis of hen's egg white with anti-egg white serum revealed 22 antigenic components.[1] Yu and Marquardt[2] in their double gel immunodiffusion pattern, observed more than one precipitin lines with single purified proteins tested against the antiserum. Similar observation was made by Yu *et al.*[3] Alfred[4] has suggested that the number of precipitin bands developing in a double gel diffusion system can be interpreted as representing the minimum number of precipitating systems present, though, however, they do not necessarily represent the maximum number.

An attempt has been made here to identify the different protein fractions on the basis of number of antigenic components present for a particular protein fraction. Since it seems feasible that a total number of 40 proteins identified so far in the egg white[5] are represented by only 22 antigenic components there must be certain common one or more than one, antigenic components for some of the proteins. This aspect has been discussed in this communication.

Materials and Methods

The antisera against purified fractions (homogenous on polyacrylamide gel electrophoresis and ultra-centrifugation at 105,000 × g) of ovalbumin, lysozyme, ovomucoid (trypsin inhibitor from egg white) and conalbumin 1 and conalbumin 2 (ovotransferrins) were developed as described by Palmitter *et al.*[6] for ovalbumin, each in two male albino rabbits of about 1 year age. Five milligram of antigen in 1 ml of saline was homogenised with 1 ml of complete Freund's adjuvant (Difco laboratories) and injected subcutaneously into the toepads of rabits. The blood was collected from heart after 2 weeks of the injection and later, serum was separated and its immunogenic specificity was tested on Ouchterlony double gel diffusion plates using 1 per cent bacto-agar (Difco) in normal saline. Goat antisera against avidin was obtained through the courtesy of Dr. A. R. Means from Campbell laboratories, USA. All the available egg white fractions were tested against the six antisera. The patterns were allowed to develop for 48 hr and the observations were recorded.

Result and Discussion

The double gel immunodiffusion patterns on Ouchterlony plates using the prepared rabbit antiserum for conalbumin 1, conalbumin 2, ovalbumin, ovomucoid and lysozyme and that of goat antiserum for avidin are presented in Fig 1 for the native egg albumen from fresh hen's egg, conalbumin 1, conalbumin 2 and ovalbumin antigens. Similar pattern for ovomucoid, lysozyme and avidin antigens are presented in Fig 2 (A to C). The avidin antiserum was also tested against all the above six antigens and its pattern is presented in Fig 2 (D). However, it may be noted that some precipitin lines are not very clearly demarcated in the figures. The minimum number of anitgenic components as shown by the number of precipitin lines in native egg albumen and in different pure fractions against the antisera tested are presented in Table 1.

In the native egg albumen the minimum antigenic components for conalbumin 1, conalbumin 2, ovomucoid and avidin were 3 each and that for ovalbumin and lysozyme 2 each. Conalbumin 1 and conalbumin 2 were similar in their antigenic properties except that conalbumin 1 showed one additional antigenic component with ovomucoid antiserum. The ovalbumin did not react with the lysozyme and avidin antiserum and showed

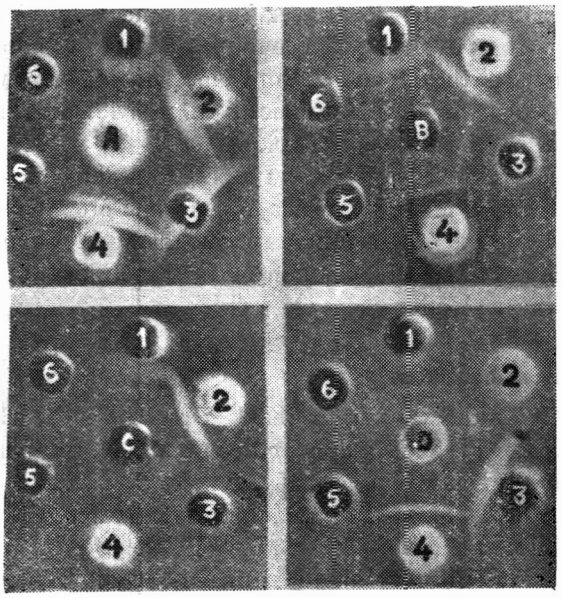

Fig. 1 Peripheral wells 1 to 6 contained antisera for conalbumin 1, conalbumin 2, ovalbumin, ovomycoid, lysozyme and avidin respectively. Central well contained the antigen.

A-Native egg albumen; B-Conalbumin 1; C-Conalbumin 2; D-Ovalbumin.

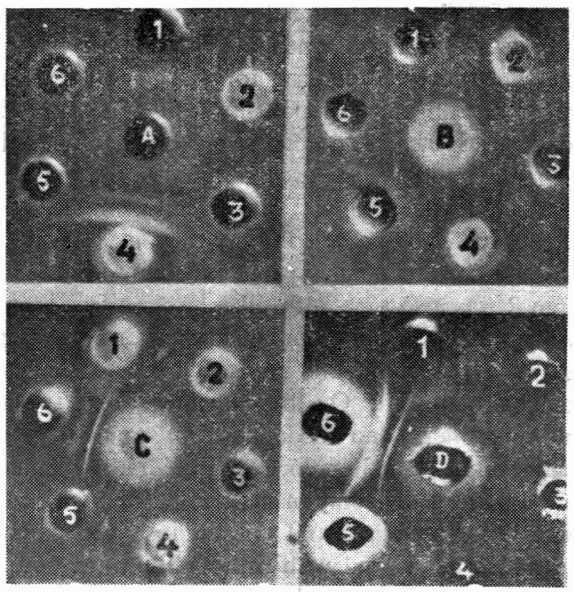

Fig. 2 A, B and C: Peripheral wells 1 to 6 contained antisera for conalbumin 1, conalbumin 2, ovalbumin, ovomucoid, lysozyme and avidin respectively. Central well contained the antigen.

A-Ovomucoid; B-Lysozyme; C-Avidin; and D-Peripheral wells 1 to 6 contained antigens—Conalbumin 1, conalbumin 2. ovalbumin, ovomucoid, lysozyme and avidin, respectively, Central well contained the goat antisera against avidin.

only one precipitin line each with conalbumin 1, conalbumin 2 and ovomucoid and two lines with ovalbumin antiserum.

The ovomucoid showed two precipitin lines with the antisera for conalbumin 1, conalbumin 2 and avidin and one each with that of ovalbumin and lysozyme. With ovomucoid antisera three preciptin lines were present. The lysozyme did not react with any other antisera expect that for lysozyme and presented two preciptin lines. The avidin showed precipitation only with ovomucoid, lysozyme and avidin antisera presenting one, one and three lines respectively.

The healthy rabbit serum did not contain any property of precipitating any of the egg white fractions. The number of antigenic components present was determined from the precipitin bands developed in the double gel diffusion test, which represent the minimum number of precipitating systems present although they do not necessarily represent the maximum number[4].

Based on this, in the immunodiffusion pattern of native egg albumen we found that a minimum of 3 precipitating systems or antigenic components are pres-

TABLE 1. MINIMUM NUMBER OF ANTIGENIC COMPONENTS PRESENT IN THE PURE FRACTIONS AS SHOWN ON DOUBLE GEL IMMUNODIFFUSION AGAINST KNOWN ANTISERA

Antigen tested	Minimum number of antigenic components as represented by number of precipitin lines present against the antisera for					
	Conalbumin 1	Conalbumin 2	Ovalbumin	Ovomucoid	Lysozyme	Avidin
Native egg albumen	3	3	2	3	2	3
Conalbumin 1	3	3	1	2	—	1
Conalbumin 2	3	3	1	1	—	1
Ovalbumin	1	1	2	1	—	—
Ovomucoid	2	2	1	3	1	2
Lysozyme	—	—	—	—	2	—
Avidin	—	—	—	1	1	3

N.B.: Reaction with healthy rabbit serum was nil.

ent for conalbumin 1, conalbumin 2, ovomucoid and avidin and 2 such components for ovalbumin and lysozyme which received confirmatory support from similar number of antigenic components seen against the pure fractions. Some of our other interesting observations are that conalbumin 1 and conalbumin 2 showed identical reaction and are thus antigenically similar though electrophoretically and chemically they are two seperate fractions. Identical reaction of conalbumin and conalbumin 2 was also seen with other protein fractions but the identical antigenic components present were either one as with ovalbumin and avidin antisera or two as with ovomucoid antisera. Ovalbumin showed only two antigenic components and one identical precipitin band with conalbumin 1, conalbumin 2 and avidin. Ovomucoid showed 3 antigenic components and reacted with conalbumin 1, conalbumin 2 and avidin antisera to show two precipitatin bands and with ovalbumin and ovomucoid antisera one precipitin band in the system. The lysozyme showed only two precipitin bands with its antisera and did not show identical reaction with antisera for any other fraction. Avidin showed three precipitin bands with its antisera and one each with ovomucoid and lysozyme. Avidin antisera when tested together with all antigens showed a similar pattern and reacted with all except ovalbumin and lysozyme. This meant that the antigenic component present in goat antiserum against avidin which reacted with other egg white fractions was not a species specific component of goat serum but rather was a true precipitating system against the egg white fractions.

Oka and Schimke[7,8], Yu *et al.*[3] and Yu and Marquardt[2] have also used double gel diffusion for the identification of egg white fractions. In the pattern presented by Yu *et al.*[3] and Yu and Marquardt[2] the multiple antigenic behaviour is seen. Further the observations of Rodriguez-Burgos and Oteiza[1] lend support to our view of a multiple antigenic component system as they found 22 antigenic components in the hen's egg white while chemically 40 individual fractions from egg white are available[5].

It appears, therefore, that at least one antigenic component is common to the major egg white protein except for lysozyme which is more specific than others and this common antigenic component is responsible for the identical reaction.

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Deacidification of High Acid Grape Musts and Wine Making with *Schizosaccharomyces pombe*

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Deacidification of high acid musts of eight varieties of grapes was carried out with *Schizosaccharomyces pombe*. *Saccharomyces cerevisiae* was also used as control. All the wines fermented with *Sch. pombe* showed considerable reduction in acidity as compared to wines fermented with *S. cerevisiae*. Absence of malic acid in wines fermented with *Sch. pombe* indicated that the malic acid was completely metabolised and this resulted in the reduction of acidity.

Acidity of the grapes is one of the criteria which determine the suitability of the grape variety for wine making. Since the flavour of the wines depends upon the optimum acidity of the grapes, high or low acid grapes are undesirable for wine making. The high acidity in grapes can be reduced either by chemical or biological agents. It is suggested that the chemical deacidification may alter the delicate flavour of the wine.[1] Therefore, biological deacidification appears to be the method of choice. Among the biological methods, the malo-lactic fermentation where the malic acid (a dicarboxylic acid) is converted to lactic acid (a monocarboxylic acid) by certain lactic acid bacteria has been widely employed in many countries to reduce the acidity of wines but this fermentation cannot take place in grape musts[2] with less than 3.3 pH[2]. Biological deacidification with *Schizosaccharomyces pombe* which is known to metabolise both glucose and malic acid to alcohol and CO_2 appears to be promising and is being currently investigated for deacidification of high acid musts.[1,3] Therefore, the use of *Sch. pombe* for deacidification of high acid grapes and wine making was investigated and the results are reported here.

Materials and Methods

Organisms: The yeast *Sch. pombe* used in this investigation was obtained from Dr. R. E. Kunkee, Department of Viticulture and Enlogoy, University of California, Davis, U.S.A. *Saccharomyces cerevisiae* which was used as control was the 'Montrachet' strain obtained from Department of Microbiology, Haryana Agricultural University, Hissar, Haryana. These cultures were maintained on yeast extract-peptone-glucose agar.

Vinification procedure: Eight varieties of grapes namely 'Alicante Bouschet', 'Seibel-8357', 'Malbec', 'Malvasia Bianca Di Wapolis', 'Isabella', 'Delaware', 'Rubired' and 'Bangalore Blue' grown in the Experimental Farm of Indian Institute of Horticultural Research, Hessaraghatta were used. They were harvested in February-March, 1977. Since the brix readings of these grapes were low, they were ameliorated with cane sugar to about 22° Brix. The grapes were crushed, sulphited with potassium metabisulphite to give 100 ppm SO_2 and after 4 hr, the musts were inoculated with 2 per cent (V/W) of respective yeast cultures. The fermentation temperature was maintained at 20 ± 1°C. After 4-5 days, the grapes were pressed and after reaching dryness, the wines were racked. The wines were then stored in glass bottles at 15°C and analysed after four months.

Chemical analysis: All chemical analyses such as total titratable acidity, volatile acidity and malic acid (by paper chromatography) were determined by the methods described by Amerine and Ough[4]. The pH was measured with Elico pH meter (Model LI-10). Brix readings were made with the help of Brix hydrometer.

Results and Discussion

Changes in pH: The pH of musts of all the grape varieties were low ranging from 3.0 to 3.25 (Table 1). The data also indicate that pH of these musts is too low for malo-lactic fermentation to occur and special efforts must be made to induce it. The initial pH of musts and final pH of wines fermented with *S. cerevisiae* and *Sch. pombe* indicate that the wines fermented with *Sch. pombe* showed an increase in pH which is due to the metabolism of malic acid. Though there is slight increase in pH of the wines fermented with *S. cerevisiae*, the *Sch. pombe* fermented wines had consi-

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TABLE 1. CHANGES IN pH AFTER FERMENTATION BY *Saccharomyces cerevisiae* AND *Schizosaccharomyces pombe*

Variety	Initial pH	Final pH	
		S. cerevisiae	*Sch. pombe*
Alicante Bouschet	3.15	3.25	3.55
Seibel-8357	3.00	3.05	3.20
Malbec	3.20	3.25	3.60
Malvasia Bianca Di Wapolis	3.15	3.05	3.20
Isabella	3.05	3.05	3.20
Delaware	3.20	3.25	3.35
Rubired	3.25	3.45	3.60
Bangalore Blue	3.10	3.20	3.30

derably higher pH. The difference in pH between must and wine fermented with *Sch. pombe* was maximum (0.40) with 'Alicante Bouschet' and 'Malbec' and minimum (0.05) with 'Malvasia Bianca Di Wapolis'. The difference in pH with other varieties were within this range.

Changes in titratable acidity: Except 'Delaware', all the varieties had acidity too high for making good quality wine. The acidity ranged from 0.87 to 1.75 (Table 2). The total titratable acidity of the musts and wines fermented with *S. cerevisiae* and *Sch. pombe* indicate that the wines fermented with *S. cerevisiae* also showed some reduction in titratable acidity. This reduction in acidity may be due to both metabolism of malic acid and precipitation of potassium acid tartarate. Malic acid is known to be metabolised to the extent of 10 to 30 per cent during alcoholic fermentation. Also, potassium acid tartarate which is present in the grapes precipitated during and after fermentation[5].

TABLE 2. CHANGES IN TITRATABLE ACIDITY AFTER FERMENTATION BY *Saccharomyces cerevisiae* AND *Schizosaccharomyces pombe*

Variety	Initial acidity (as g. tartaric acid/100 ml.)	Final acidity (g/100 ml)			
		S. cerevisiae	% less	*Sch. pombe*	% less
Alicante Bouschet	1.07	0.835	22	0.460	57
Seibel-8357	1.75	1.190	32	0.745	58
Malbec	1.39	0.971	30	0.478	66
Malvasia Bianca Di Wapolis	1.02	0.950	7	0.592	42
Isabella	1.17	1.078	8	0.642	45
Delaware	0.87	0.785	10	0.680	22
Rubired	1.19	0.946	20	0.650	45
Bangalore Blue	1.10	1.053	4	0.835	24

However, the *Sch. pombe* fermented wines had considerably lower titratable acidity than *S. cerevisiae* fermented wines. The difference in titratable acidity between the must and wine fermented with *Sch. pombe* was maximum (66 per cent), in case of 'Malbec' and minimum (22 per cent) with Delaware'. Other wines were within this limit. The difference in acid reduction within the varieties may be due to difference in amount of malic acid present in grapes. It is also seen that very high acidity in musts of 'Seibel-8357' and 'Malbec' were considerably reduced.

Malic acid in wines: Presence or absence of malic acid in wines after fermentation with *S. cerevisiae* and *Sch. pombe* was determined by paper chromatography. It was found that all the wines made by fermentation with *S. cerevisiae* showed the presence of malic acid. Except the wines from three varieties namely 'Delaware', 'Rubired' and 'Bangalore Blue', the wines from other varieties fermented with *Sch. pombe* showed the absence of malic acid. *Sch. pombe* fermented wines from these three varieties showed a mild spot indicating the presence of small amount of malic acid. Since *Sch. pombe* is a slow grower, the fermentation of musts from these three varieties by this yeast might have been inhibited by competition from the natural flora of yeasts present on the grapes, resulting in presence of small amount of malic acid which gave a mild spot. Similar observation was made by Yang[1] who also gave same reason for the presence of malic acid in some wines.

The reason given above for the presence of malic acid in some wines might be true since the fermentation of these three varieties were initiated not by pure culture of *Sch. pombe* but by addition of inoculum from fermenting batches which were inoculated with *Sch. pombe* previously. This inoculum might be containing both *Sch. pombe* and natural yeasts. In order to establish that the limited reduction in acidity was due to competition from natural yeasts, musts from two varieties namely 'Rubired' and 'Bangalore Blue' harvested during August, 1977 were inoculated with pure culture of *Sch. pombe*. Paper chromatographic analysis indicated that the *Sch. pombe* fermented wines did not show any malic acid spot while *S. cerevisiae* fermented wines showed the spot. The result of this experiment supports the suggestion made earlier that the limited reduction in acidity was due to competition from natural flora of yeasts. It also suggests that use of pure inoculum of *Sch. pombe* is very important in deacidification studies, since contamination of natural yeasts may inhibit the growth of *Sch. pombe* and result in limited deacidification.

The flavour and odour of the wines fermented with *Sch. pombe* and *S. cerevisiae* were almost the same. Organoleptic analysis of the wines showed that the wines fermented with *Sch. pombe* were less acid than the wines fermented with *S. cerevisiae*. However, the wines made from 'Alicante Bouschet' and 'Malbec' with *Sch. pombe* had acidity lower than the optimum required for table wines. Though the acidity of these wines can be corrected by blending, ways to keep the acidity of wines within the optimum range after fermentation by *Sch. pombe* is being investigated and will be reported soon.

Acknowledgement

The authors are grateful to Dr. G. S. Randhawa, Director, Indian Institute of Horticultural Research, Bangalore, for his interest in this study. They are also grateful to Dr. Rajendra Singh, Geneticist (Grapes) for supplying the grape varieties.

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Corrosion of Tinplate Cans by Vegetables - Composition of Ivy Gourd (*Coccinia indica* Wright & Arn.) with Special Reference to Corrosion Accelerating and Inhibiting Compounds

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As a prelude to understand the unusual tinplate corrosive action of ivy gourd, an attempt is made to analyse its constituents. This vegetable is found to be accumulating nitrate to high levels. The distribution of nitrate in the vegetable along its longitudinal axis increases steeply towards the stem end from the stylar end. The concentration of nitrate in the exocarp is about 2.6 times that in the mesocarp. Nitrite in the tissue of the fresh vegetable is very low but increases during improper handling and storage. The nitrate reductase activity in the vegetable has an inverse relationship to the nitrate content. Light radiations activate the nitrate reductase enzyme system and decrease the nitrate content. The free amino acid pool in the vegetable is rich in arginine, serine, glutamic acid, threonine and alanine. It contains a peptide which has a component resembling cysteic acid. About 70% of the copper in the vegetable is mainly associated with the protein fraction and is predominantly present in its higher valency state. About 60% of the lipids are amphiphilic in nature.

Ivy gourd (*Coccinia indica* Wright & Arn.) is a highly corrosive vegetable when canned, eventhough its pH is 4.5 to 4.8. Some earlier workers[1] have attributed the corrosive nature of this vegetable to the organic acids, particularly to malic acid. The corrosive action of organic acids like oxalic acid is shown by Sherlock and Britton[2] to be due to the high stability constants of their tin complexes. The stability constants of the complexes of citric and malic acids are reported by them to be relatively low. Hence, in the absence of oxalic acid in ivy gourd, the other organic acids do not fully account for the extent of corrosion exhibited by the vegetable.

Polyphenolic constituents have been shown to function as depolarisers by some workers.[3,4] But according to some others[5], polyphenols do not affect the corrosion mechanism at all.

Degradation products of ascorbic acid like dehydroascrobic acid, 2-3, diketo gulonic acid and furfural have been shown to be tinplate corrosion accelerators[6]. Hope[7] reported that ascorbic acid added to apple halves controlled the container from corrosion.

Some amino acids like cysteine and penicillamine have been reported to behave like organic acids forming tin complexes and when the ratio of stannous ions to ferrous ions is lower than the value 5×10^{-11}, tin becomes more electronegative than iron, which can accelerate corrosion[8].

Nitrate is the normal source of nitrogen absorbed by plants and according to Kruger[9] accumulation of nitrate implies that conversion has not kept pace with assimilation. Walker has reported the nitrate contents of several vegetables and fruits[10].

No work has been carried out in India on the accumulation of nitrate in vegetables and fruits and its possible role in tinplate corrosion. This paper presents the first part of a series of investigations into these aspects. This deals with the detailed analysis of ivy gourd with specific reference to compounds which may have some role in tinplate corrosion.

Materials and Methods

Ivy gourd: Fresh tender ivy gourd of canning maturity grown near Mysore city, India, was used in this study. The analysis of ivy gourd for a particular year and season was carried out on a single batch. Ivy gourd was freeze dried, powdered and stored at—18°C and required quantities were drawn whenever necessary. The analytical data were finally computed on freshweight basis. For some estimations ivy gourd of different maturities were also used.

Moisture, ash, crude fibre, protein, crude fat, titrable acidity, calcium and phosphorus were determined by the A.O.A.C. methods.[11] The following analyses were also carried out: sugars,[12] tannin,[13] pectin,[14] ascorbic acid,[15] iron,[16] copper,[17] valency state of copper,[18] molybdenum,[19] chlorophyll[20].

Measurement of pH: This was done using a Toshniwal pH meter having a combination electrode.

Free amino acids: Free amino acids were separated from other constituents by the method followed by El-Mansy and Walker[21]. Total amino acids in the sample was determined by the method of Moore and Stein[22] and expressed as leucine. Quantitative analysis of individual amino acids was carried out with a Beckman 120 B amino acid analyser under conditions recommended for physiological fluid analysis[23].

High voltage electrophoresis: The free amino acid fractions expected to contain peptides were hydrolysed with 6N electrolytically pure hydrochloric acid, acid removed and the amino acids were separated by high voltage electrophoresis[24].

Organic acids: Organic acids were separated by the method of El-Mansy and Walker.[21] Paper chromatographic separation and identification were carried out according to the procedure of Hulme[25]. For the quantitative estimation of organic acids, gradient ion-exchange chromatography employed by Hulme and Wooltorton was adopted[26].

Fractionation of alcohol insoluble residue: Pectic substances were extracted by the method of Devar *et al.*[27] Protein was extracted with 0.1N sodium hydroxide and precipitated with 10 per cent trichloro acetic acid. The residue left after extracting pectin and protein was taken as crude fibre.

Nitrate and nitrite: Nitrate was detected by the diphenyl amine spot test[28]. Nitrate and nitrite were estimated by the method of Eipeson *et al*[29].

Nitrate reductase activity: Nitrate reductase activity was determined according to the method followed by Luh[30].

Nitrate distribution in the vegetable: Ivy gourd was cut longitudinally and freeze dried. The exocarp and mesocarp portions were separated and nitrate contents determined. To determine the distribution of nitrate along the longitudinal axis, ivy gourd was frozen quickly, sliced into about 2 mm thick slices, weighed and the nitrate content in the slices determined.

Lipids: Lipids were extracted and purified from water soluble contaminants by Folch's method.[31] The total lipid was fractionated into netural glyco and polar fractions by the method described by Nagy *et al.*[32] Thin layer chromatographic separation of the lipids were effected by several solvent systems suggested by Stahl, Marinetti, Nichols and Kates[33-37] and identified by the specific spray reagents suggested by them.

Fatty acids: Fatty acids after converting to their fatty acid methyl esters[38] were separated by gas liquid chromatography using a varient aerograph-1400.

Results and Discussion

Table 1 gives the proximate composition of ivy gourd analysed during two seasons and two years. It is seen that in general the seasonal variations in the chemical composition are not very significant. The results show that ivy gourd is a potential nitrate accumulating vegetable having a wide variation in its content (90 to 320 ppm). Nitrite content in the fresh vegetable is very low (0.2 to 0.4 ppm). This is in agreement with earlier reports[10].

Analysis of organic acids in ivy gourd shows that about 80 per cent of the organic acids is constituted of citric, malic and succinic acids. The minor acids detected are shikimic, quinic, glyoxylic, fumaric and α-ketoglutaric acids. Table 2 gives the changes in concentrations of the major organic acids during maturation. It is evident that as the fruit matures, the citric acid content increases steadily. As the stability constant of tin complex with citric acid is higher than that of malic acid[2] it can be expected that the corrosivity of ivy gourd will increase as the maturity increases.

Table 3 gives the free amino acid composition of ivy gourd. It is seen that the amino acid pool is rich in arginine, serine, alanine and γ-aminobutyric acids. Acid hydrolysed amino acid fraction on high voltage

TABLE 1. PROXIMATE CHEMICAL COMPOSITION OF IVY GOURD

Constituent	April		September	
	I Year	II Year	I Year	II Year
Exocarp %	55	56	56	56
Mesocarp %	45	44	44	45
Moisture (Exocarp) %	92.5	92.4	93.5	93.7
Moisture (Mesocarp) %	92.3	92.5	93.8	93.6
Moisture % (whole)	92.5	92.4	93.6	93.7
Protein %	1.24	1.25	1.22	1.20
Crude fat %	0.09	0.11	0.09	0.10
Total sugar %	1.64	1.69	1.60	1.54
Reducing sugar %	1.35	1.40	1.32	1.30
Crude fibre %	1.62	1.65	1.70	1.69
Ash %	0.67	0.61	0.63	0.60
Calcium (mg/100 g)	39	41	48	37
Iron (mg/100 g)	1.5	1.5	1.4	1.3
Phosphorus (mg/100 g)	32	31	30	31
Copper (mg/100 g)	0.06	0.07	0.06	0.06
Molybdenum (mg/100 g)	0.04	0.04	0.04	0.04
Tannin (mg/100 g)	42.5	48.3	40.2	40.4
Pectin %	1.4	1.2	1.2	1.1
Organic acids (as citric acid %)	0.11	0.11	0.11	0.10
pH	4.9	4.8	5.0	5.2
Ascorbic acid (mg/100 g)	24	22	26	24
Chlorophyll (mg/100 g)	0.96	1.02	1.12	1.15
Free amino acids (as leucine) m.mole/100 g.	1.35	1.50	1.40	1.45
Anions (m.equiv/100 g)	8.6	8.5	8.3	8.4
Nitrate (mg/100 g)	10-30	10-32	9-28	9-29
Nitrite (mg/100 g)	0.03	0.04	0.04	0.02

electrophoretic separation showed a spot corresponding to cysteic acid.

Table 4 gives the distribution of nitrate in the exocarp and mesocarp portions of ivy gourd samples of different nitrate contents. It is observed that even in those widely varying nitrate containing ivy gourd samples, the exocarp to mesocarp ratio for nitrate is about 2.6.

Fig. 1. shows the distribution of nitrate from the stem end to the stylar end of the vegetable. This clearly

TABLE 2. CHANGES IN THE ORGANIC ACID COMPOSITION OF IVY GOURD DURING MATURATION

Organic acid	Milli equivalents/100 g (1:1)*		
	Tender	Normal	Ripe
Succinic acid	4.3	4.1	3.9
Malic acid	2.4	2.1	1.6
Citric acid	0.2	0.7	1.6

*Twice diluted to represent the canned product

TABLE 3. FREE AMINO ACID COMPOSITION OF IVY GOURD

Amino acid	μ moles/100 g.
Lysine	18.3
Histidine	40.0
Arginine	283.5
Aspartic acid	56.5
Threonine	115.0
Serine	424.0
Glutamic acid	149.5
Proline	24.5
Glycine	57.5
Alanine	232.5
Cystine	traces
Valine	26.0
Isoleucine	10.5
Leucine	31.0
Tyrosine	6.0
Phenylalanine	19.0
γ-amino butyric acid	50.0
Hydroxyproline	traces

TABLE 4. DISTRIBUTION OF NITRATE IN THE EXOCARP AND MESOCARP OF DIFFERENT IVY GOURD SAMPLES

Particulars	Nitrate content (ppm)					
Exocarp	355	300	248	224	185	165
Mesocarp	135	115	92	90	72	63
Ratio	2.6	2.6	2.7	2.6	2.6	2.6

TABLE 5. NITRATE, NRA AND MOLYBDENUM CONTENT IN IVY GOURD

Nitrate (ppm)	NRA (moles No_2/hr)	Molybdenum (ppm)
52	0.35	0.62
85	0.33	0.60
108	0.34	0.56
130	0.30	0.60
160	0.28	0.48
210	0.26	0.42
240	0.22	0.40
280	0.20	0.51
340	0.19	0.52
406	0.15	0.35

TABLE 6. EFFECT OF DIFFERENT LIGHT RADIATIONS ON THE NITRATE AND NITRITE CONTENTS AND NRA OF IVY GOURD

Source of light radition	Exposure time (hr)	Nitrate (ppm)	Nitrite (ppm)	NRA
60 Watts	—	195	0.30	0.27
tungsten lamp	2	190	0.25	0 30
	4	185	0.35	0 35
	6	165	0.28	0 42
	8	145	0.30	0 40
Sunlight	—	195	0.30	0 27
(26 to 30°C)	2	175	0.28	0 50
	4*	166	0.32	0.52
	6	138	0.25	0.49
	8	118	0.28	0.52

*The fruits started shrivelling

TABLE 7. EFFECT OF STORAGE, HANDLING AND OTHER VARIABLES ON THE NITRITE CONTENT OF IVY GOURD

Storage, handling and other variables	Storage period (days)	Storage temp. (°C)	Nitrite (ppm)
Fresh	—	—	0.25
Stored without injury	5*	20-25	2.60
Stored after injuring**	4*	20-25	10.4
Cut longitudinally into pieces	2*	20-25	20.6
Blended in Waring blender	1	20-25	48 5

*Microbial growth was visible afterwards.

**The fruits were injured by slight hit with a wooden hammer.

TABLE 8. PERCENTAGE COMPOSITION OF NEUTRAL GLYCO AND POLAR LIPIDS OF IVY GOURD

Lipids	% composition
Neutral	42-46
Glyco	18-21
Polar	36-40

TABLE 9. RELATIVE PERCENTAGES OF FATTY ACIDS IN THE GLYCO AND POLAR LIPID FRACTIONS OF IVY GOURD

Fatty acid	Glyco lipid %	Polar lipid %
C 16:1	48.7	38.6
C 18:0	18.2	8.9
C 18:1	8.7	4.3
C 18:2	14.7	24.8
C 18:3	9.7	23.4

indicates the possibility of reducing the nitrate content indicates by trimming the stem end to a larger extent.

The molybdenum contents and nitrate reductase activities (NRA) of ivy gourd samples of different nitrate contents are given in Table 5. There is an inverse relationship between nitrate content and nitrate reductase activity. However, no correlation between nitrate contents and molybdenum contents could be established even though it is known that molybdenum is one of the prosthetic groups of the nitrate reductase system.

It is seen from Table 6 that sunlight activates nitrate reductase effectively and reduces the nitrate content. This finding suggests a possibility to reduce nitrate content and also to reduce can corrosion.

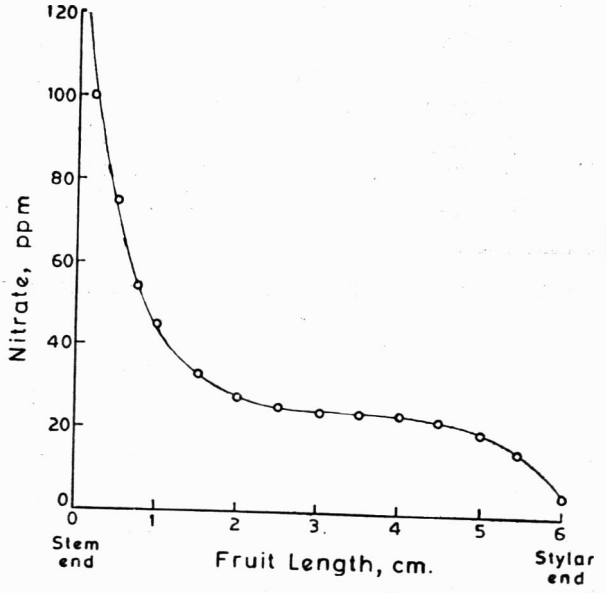

Fig. 1. Longitudinal Distribution of Nitrate in Ivy Gourd.

Table 7 gives the effect of storage and handling variables on the nitrite content of ivy gourd. The results show that injuring the vegetable can increase the nitrite content very high. Nitrite being a very strong detinner can cause heavy corrosion.

About 70 percent of copper in the vegetable was found to exist in the higher valency state. Thermodynamically this has extreme emportance in the corrosion process of tinplate containers.

The lipids in ivy gourd was found to be constituted of about 42-46 percent neutral, 18-21 per cent glyco and 36-40 per cent polar lipids (Table 8). Table 9 shows the relative percentages of fatty acids in the glyco and polar lipid fractions. Based on certain surface properties the amphiphilic lipids can manifest, it can be presumed that they may have important roles in the tinplate corrosion mechanism. The low level (22 to 24 mg/100 g) of ascorbic acid present in the vegetable does not appear to have any significant role on corrosion.

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Survey of Quality of Milk-Products in Bombay

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Survey of chemical quality of milk products like *chakka*, *shrikhand*, *khoa*, *burfi*, *pedha*, *gulab Jamun*, *rasogolla*, table-butter, *desi*-butter and *ghee* in Bombay and greater Bombay markets has revealed that adulteration is rampant, unscruuplous practices like addition of cheaper ingredients to *khoa* based confectionaries, keeping high percentages of moisture, using inferior quality ingredients, addition of vegetable fats to table-butter, *desi*-butter and ghee and addition of fillers to *Chakka*, *shrikhand* and confections are very common.

About 49 per cent of the milk produced in India is converted into *ghee*, 9 per cent used for preparing fermented milk products like *dahi*, 6 per cent for production of *khoa*, the base of Indian confectionary and about 2 percent for production of other indigenous products like *malai*, *rabari*, *khurchan*, etc[1]. Despite the laws governing the control of quality and sale of various milk products, adulteration of milk-products has not been possible to check completely. The problem arises from the increased demand and short supply. With a view to assess the quality of market milk products available in Bombay and greater Bombay, a survey was taken up.

Materials and Methods

The following milk products were collected from different markets of Bombay and greater Bombay. They were collected in sterlized, dried, wide mouthed glass bottles.

Chakka: This is a fermented milk product. A total of 105 market samples were collected and analysed for moisture, fat, protein, lactose and acidity. Samples of *chakka* were also prepared from cow and buffalo milk samples as per the method of Srinivasan[1] by draining off the serum of *dahi* and analysed for the above constituents.

Shrikhand: This is a fermented milk product. About 58 samples were collected from the market and analysed for different chemical constituencies as under chakka. Milk products were also prepared from cow and buffalo milks following the method described in Indian Dairy Products[15].

Khoa: This is a desiccated milk product. Based on its texture and the end use, it is being sold under various trade names like *harani*, *rawa*, *dhap*, etc. A total of 550 samples were collected and examined for various chemical constituents.

Burfi: This is prepared from *khoa*. Eighty samples were collected and analysed for moisture, fat, acidity, protein, lactose and sucrose. The different types of *burfi* collected included *Rawa* type, *Mawa* type, flavoured, dry fruit, chocolate and cocoa flavoured types.

Pedha: This is also a *khoa* based product. Twenty five market samples were collected and analysed for different chemical constituents as in Table 2.

Gulabjamun: A *khoa* based milk product sold as sweet meat. Ten samples were collected and analysed.

Rasogolla: This is a coagulated milk product. A total of 35 market samples were collected and examined.

Table butter, desi butter and ghee: A total of 136, 138 and 80 samples of table butter, *Desi* butter and ghee samples respectively collected from the market were analysed for moisture, fat, salt, curd, R. M. Value, Polenske value, acidity and FFA. Ghee is the clarified moisture free fat obtained from butter.

Butter and ghee samples were analysed as per the ISI methods[2-4] where as other products were analysed as per the AOAC methods[5]. Samples were analysed in duplicate.

Results and Discussion

Chakka: The range in value of moisture, protein, fat, lactose and acidity of market samples and those prepared in the laboratory from cow and buffalo milk samples are given in Table 1. They varied largely from those reported by Ganguli[6]. The market samples contained higher moisture (max. 83.2 per cent) and some samples contained negligible amount of fat (0.50 per cent) as compared to laboratory sample whose fat content did not go below 10.5 per cent. Much variation was not found in market samples and laboratory samples with respect to protein, lactose and acidity.

TABLE 1. RANGE IN VALUES OF MARKET SAMPLES OF *chakka*, *Shrikhand*, *khoa* AND *rasogolla*

Samples	Moisture %	Fat %	Protein %	Lactose %	Sucrose %	Acidity % (Lactic)
			Chakka			
Market	57.5–83.2	0.5 –27.0	7.8 –17.1	0.08– 2.48	—	0.60–2.95
Laboratory prepared						
Cow milk	60.0–74.4 (67.7)	10.5 –16.6 (13.4)	10.8 –16.1 (12.4)	0.89– 2.56 (1.77)	— —	0.88–2.75 (1.79)
Buffalo milk	60.7–69.5 (67.2)	13.2 –18.3 (15.7)	10.2 –14.4 (12.5)	0.87– 1.86 (1.30)	— —	1.20–2.35 (2.07)
			Shrikhand			
Market	25.4–40.8	4.5 –11.4	3.4 –15.7	0.66– 2.79	38.8–57.10	0.50–2.16
Laboratory prepared						
Cow milk	29.1–38.4 (33.8)	4.50–10.00 (7.3)	4.25– 8.05 (6.2)	1.05– 1.90 (1.50)	42.0–50.9 (46.40)	0.55–2.50 (1.50)
Buffalo milk	33.6–39.4 (36.5)	6.5–12.5 (9.5)	6.0– 9.6 (7.3)	1.00– 2.05 (1.00)	42.6–48.6 (45.6)	0.85–1.70 (1.25)
			Khoa			
Market	9.9–44.6	13.5–36.0	19.8–23.3	9.88– 29.0	—	0.28–0.38
			Rasogolla			
Market	29.7–53.4	2.0– 9.0	0.4– 5.6	0.22–17.75	16.6–59.4	0.06–0.41

Figures in the parenthesis indicate mean values.

Shrikhand: The composition of *shrikhand* obtained from market and those prepared from cow and buffalo milk in the laboratory are given in Table 1. Like *chakka*, the market samples of *shrikhand* showed large variations in their moisture, fat, lactose, sucrose and protein contents. The wide variation in moisture and sugar is an obvious indication of their being sub-standard as also reported by Upadhyay[7]. Although much variation is not seen in the moisture and fat contents between the market and laboratory prepared samples, the market samples contained surprisingly high amount of protein (15.72 per cent).

TABLE 2. QUALITY OF *Khoa* BASED INDIAN CONFECTIONARIES (MARKET SAMPLES)

Product	No. of samples	Moisture (%)	Fat (%)	Acidity % (lactic)	Protein (%)	Lactose (%)	Sucrose (%)
Burfi							
Rawa type	14	2.1–19.8	12.5–27.0	0.15– 0.48	2.6– 9.8	10.0–18.6	21.9 –43.1
Mawa „	24	4.7–20.0	8.8–26.8	0.10– 0.43	1.4–11.8	5.6–18.3	16.7 –59.7
Flavoured type	10	3.8–11.3	9.3–25.0	0.24– 0.48	2.9–11.1	9.7–14.0	28.2 –42.6
Dry fruits „	12	5.6–12.8	14.1–24.0	0.19– 0.43	3.2–10.4	10.9–20.0	21.6 –33.6
Chocolate „	10	9.0–14.2	17.0–25.0	0.21–10.37	5.3– 6.8	8.1–14.7	22.7 –40.1
Cocoa „	10	3.4– 8.8	17.9–20.0	0.28– 0.40	6.5– 5.6	13.8–15.4	28.88–28.90
Pedha	25	4.2–14.2	7.0–25.0	0.08– 0.41	1.4–12.1	4.0–18.6	13.2 –61.8
Gulabjamun	10	22.2–41.8	5.0– 8.0	0.06– 0.12	0.0– 3.4	2.6– 5.4	30.5 –41.3

TABLE 3. QUALITY OF MARKET SAMPLES OF TABLE BUTTER, *Desi Butter* AND *Ghee*

	Moisture %	Fat %	Salt % (NaCl)	Curd %	R. M. Value	Polenske Value	Acidity % (oleic)	FFA %
Table-butter	10.2–71.0	25.5–89.1	0.08–5.43	0.02–24.8	0.69–34.9	0.20–2.61	0.01–1.08	—
Desi-butter	9.6–63.6	22.7–87.6	0.05–0.88	0.18–26.8	2.06–34.8	0.45–3.10	0.05–2.13	—
Ghee	0.02–0.79	93.5–99.9	—	0.51– 6.5	1.00–34.9	0.20–1.76	—	0.01–0.38

Khoa: The moisture, fat, protein and lactose contents of market samples of *khoa* varied widely and also differed largely (Table 1) as compared to the standards prescribed by ISI[2] and PFA[8]. The market samples possessed hard granular texture with dirty white or grey appearance with bitter or saltish taste which may be due to the use of high acid milk or addition of neutralizers or stabilizers[1] or the product itself being old. Market samples of *khoa* were also found containing starch as shown by positive iodine test. This is also reported by Sharma[9].

Burfi: The large variation in its chemical constituents (Table 2) may be attributed to its being prepared under uncontrolled conditions to suit various demands of the trade. The vital parameters like moisture and sucrose which were found to touch as high as 20 and 59 per cent respectively obviously indicate the substandard conditions prevailing in its preparation as also reported by Ghodekar[10]. The market samples of *burfi* were also found having moldy flavour obviously derived from its base material (*khoa*). In such cases attempts to suppress the moldy flavour by use of added flavours were found very common.

Pedha: Wide variation was noted in the chemical constituents (Table 2). Similar findings were also reported by Ghodekar[10]. Starch was found present in many *pedha* samples as reported by Sharma[9]. Moldy and off-flavour indicates the product being prepared from stale *khoa*.

Gulabjamun: The variations in chemical composition indicate (Table 2) that the products are being prepared under uncontrolled conditions in absence of any standard. Moreover, few samples exhibited off flavour obviously derived from its substandard base material *khoa*.

Rasogolla: The chemical composition is given in Table 1. Comparing with the ISI[4] specification, 18.10 per cent of the samples were found substandard in respect of fat, 55.0 per cent in respect of protein and 18.10 per cent in respect of sucrose contents. The sucrose content ranged from 16.57 to 59.37 per cent.

Table butter: Large variations are observed in the chemical composition (Table 3) as compared to PFA[8] standards which prescribed 16.0 per cent moisture (max) 80.0 per cent fat (min), 26.0 R. M. value (min), 2.0 per cent salt (max), and 1.50 per cent curd[11] and 1.70-2.90 Polenske value[12]. Compared to the above standard, 44.2 per cent of the samples failed in respect of moisture and fat, 14.7 per cent in respect of salt (NaCl), 18.4 per cent in respect of curd and 29.4 per cent in respect of R. M. and Polenske values. Baudouine test was found positive in 29.4 per cent of the samples indicating adulteration with hydrogenated vegetable fat. It was very interesting that a few samples showed a moisture content as high as 71.0 per cent, fat as low as 25.5 per cent and R. M. value of 0.69, thereby establishing the very poor quality of the product and the use of hydrogenated vegetable oil in their preparation.

Desi butter: Samples varied largely in their moisture, fat, curd, acidity, salt contents and R. M. and Polesnke values (Table 3). As compared to the values reported by ICAR[13] and Srinivasan,[1] 44.2 per cent of the samples failed in respect of moisture and fat contents, 18.4 per cent in respect of curd content, 14.7 per cent in respect of salt content, 29.4 per cent in respect of R. M. and Polenske values. Baudouine test was found positive in 29.4 per cent of the samples. In the absence of any prescribed standard the adulteration of the product is rampant.

Ghee: Among the samples tested (Table 3), 19.0 per cent failed in respect of moisture, 25 per cent in respect of curd, 18 per cent in respect of R. M. and Polenske values, when compared with the PFA[8] and AG Mark[14] standards. Baudouine test was found positive in 20 per cent of the samples. Overall findings reveal that adulteration in *ghee* is comparatively less than that found in table butter and *desi* butter which may be due to strict quality control.

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Growth Retardant Effect of Some Indigenous Plant Seeds Against Rice Weevil *Sitophilus oryzae* (L.)

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The growth disruption effects of *Annona squamosa* (L.) and *Azadirachta indica* (Adr. Juss.) seed extracts were tested against *S. oryzae*. The insects were exposed to the surface treated wheat with *A. squamosa* seed extract and an isolate from *A. indica* seeds in glass vials. *A. squamosa* and *A. indica* prolonged the development and caused loss inbody weight among *S. oryzae*.

During the last decade, work on growth retardants has been in progress against the field pests. Chloroform extracts of persian lilac or chinaberry (*Melia azadirach* Linn.) retarded the growth and development of lepidopterous larvae like *Spodoptera frugiperda* and *Heliothis zea* (Boddie)[1]. A water extract of seeds and leaves of *Azadirachta indica* A. Juss. retarded the growth and development of *Schistocerca gregaria* (Forsk)[2]. Further, growth disruption of final instar larvae of diamond back moth (*Plutella xylostella* L.), tobacco budworm (*Heliothis virescens* Fabricus) and cotton stainer (*Dysdercus fasciatus* Signoret) has been caused by 'Azadirachtin', a highly oxidized triterpenoid isolated from seeds[2]. Against stored grain beetles, so far, only neem seed powders have been tried by mixing them with the grains as protectants[3,4]. However, in a programme of research, out of several indigenous plant materials screened, extracts of *A. squamosa* and *A. indica* seeds have shown growth disruption against *S. oryzae*.

Materials and Methods

The sources of the indigenous plant seeds were as follows: Custard apple (*Annona squamosa* Linn.) and neem (*Azadirachta indica* Adr. Juss.) belong to the families Annonaceae and Meliaceae respectively.

The seeds were powdered and passed through a 140-mesh sieve.

Ether Extracts of A. squamosa and A. indica: 1,000 g of powdered seeds from each plant species was extracted in the cold with diethyl ether. The yield of the residues on removal of the solvent was 60 and 65 g from custard apple and neem seed powders respectively. For use as candidate growth retardant custard apple seed extract, the residue was dissolved in acetone to give suitable concentrations. 1.5 g of an isolate was obtained from 2 kg seeds of neem by following the procedure as described by Butterworth and Morgan[5] for the isolation of 'azadirachtin' a highly oxidized triterpenoid ($C_{35}H_{40}O_{16}$) with fourteen of the oxygen atoms deployed in five

ester groups, three free hydroxy groups and dihydrofuran ring: the remaining oxygen atoms tentatively assigned to two ether groups. For use as candidate growth retardant, the isolates were dissolved in diethyl ether to give suitable treatments to wheat.

Rice weevil exposures and development in surface treated wheat: The necessary amount of custard apple seed extract and an isolate from neem seed for application at 0.125, 0.25 and 0.375 g was dissolved separately in diethyl ether to allow application to all exposed surfaces of each lot of 100 g wheat in a crystallizing dish by shaking it slowly. Then the diethyl ether was evaporated and the wheat was stored in glass containers. This wheat was used for insect exposures one day after treatment.

Ten gram samples were placed in glass vials measuring 12 cm in length and 3 cm in diameter. The vials were covered with pith corks. Twenty five rice weevils, 1 to 4 week old were introduced to investigate the effects of custard apple seed extract and an isolate from neem seed on the growth of rice weevil larvae, pupae and adults. The development of the larvae, pupae and adults were observed upto the emergence of control adults. Therefore, 21 days after the insects were introduced, all insects were removed. When F_1 progeny emergence started, the adults were counted and removed at every 2 or 3 days. Counting was terminated 15 days after the 1st emergence to avoid F_2 progeny.

Results and Discussion

Growth retardency among S. oryzae: Relative efficacies of the growth retarding materials against *S. oryzae* are shown in Table 1.

From Table 1, it is clear that the insects exposed to treated wheat developed at a much slower rate than the controls and the larvae, pupae and adults were also smaller in size.

Table 2 shows a significant effect on the body weight of the larvae, pupae and adults collected from the neem and custard apple treated lots of wheat as compared to insects collected from wheat treated with diethyl ether only. This effect on the body weight was much more pronounced among the larvae, pupae and adults collected from the custard apple treated wheat as compared to neem treated wheat.

The custard apple seeds are known to possess some insecticide as effective as rotenone[6]. Maddrell and Casida[7] showed that insecticide of plant origin like nicotine caused paralysis and the accumulation of large volumes of rectal fluid leading to diuresis among insects. It is, therefore, possible that the growth disruption effects of custard apple seed extract are due to the release of diuretic hormone into the haemolypmh of larvae, pupae and *S. oryzae*, which is evident due to loss in body weight. Hence, growth disruption properties of custard apple seed extract could be used in pest control programmes as initiators of disadvantageous excretion among pest species.

TABLE 1. EFFECTS OF CUSTARD APPLE SEED EXTRACT AND OF AN ISOLATE FROM NEEM SEEDS ON THE DEVELOPMENT OF *S. oryzae* ON 34TH DAY OF EXPOSURE

Material	% concn. (ppm)	Emergence of			Total emergence
		Larva	Pupa	Adult	
Custard apple	3750	20	11	19	50
Neem	3750	32	13	15	60
Custard apple	2500	33	20	37	90
Neem	2500	37	30	66	133
Custard apple	1250	34	20	89	143
Neem	1250	27	38	84	149
Control	—	20	34	177	231

TABLE 2. EFFECTS OF CUSTARD APPLE SEED EXTRACT AND OF AN ISOLATE FROM NEEM SEEDS ON THE BODY WEIGHT OF LARVAE, PUPAE AND *S. oryzae*

Material	Stage of development	% concn (ppm)	Av. wt* of insects (mg)	% reduction in wt.
Custard apple	Larvae	1250	1.5	16.7
Neem	(3rd instar)	1250	1.7	5.6
Control	,,	ET	1.8	—
Custard apple	Pupa	1250	2.0	5.0
Neem	,,	1250	2.1	4.5
Control	,,	ET	2.2	—
Custard apple	Adult	1250	1.3	27.8
Neem	,,	1250	1.4	22.2
Control	,,	ET	1.8	—
Custard apple	Larva	3750	1.2	33.3
Neem	(3rd instar)	3750	1.3	27.8
Control	,,	ET	1.8	—
Custard apple	Pupa	3750	1.6	27.3
Neem	,,	3750	1.9	13.6
Control	,,	ET	2.2	—
Custard apple	Adult	3750	1.4	22.2
Neem	,,	3750	1.4	22.2
Control	,,	ET	1.8	—

*From 3-4 replicates; in each replicate 10 individuals were weighed. ET: Ether treated.

The isolate from neem seeds is known to possess some of the features of insect ecdysones.[2] Robbins *et al.*[8] showed that ingestion of a number of ecdysone analogues gave inhibition of insect growth and development, for example in *Manduca sexta* Joh., the tobacco horn worm. It is, therefore, possible that the growth disruption effects of an isolate from neem is due to interference with the normal hormone balance of the insects. Hence, could be employed as a control agent against a number of stored products pests.

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RESEARCH NOTES

COMPARISON OF THREE METHODS FOR DETERMINING PROTEIN CONTENT IN WHEAT

Three methods of protein estimation in wheat namely, Kjeldahl, dye binding and Infra red reflectance were compared by testing 222 samples of wheat. The results were identical within the limits of precision obtained by these methods. The values obtained by dye binding capacity and Infra red analysis were lower as compared to Kjeldahl method. Statistical analysis of the data revealed that the differences in values for protein content by three methods are non-significant, indicating the suitability of any of the method.

There is a need for a rapid and accurate method to provide information about protein content of food stuffs at a low cost and on the spot. For this, several methods are in use such as Kjeldahl nitrogen determination,[1] dye binding capacity,[2] biuret,[3] alkaliphenol reaction,[4] neutron activation,[5] micro diffusion technique,[6] Folin-Phenol reagent,[7] Nessler's reagent[8] and Grain Quality Analyzer[9]. These methods suffer from one or the other drawbacks such as cost, time needed for analysis, narrow range of application and not useful for rapid screening.

Watson *et al.*[10] reported highly significant correlation coefficients between Kjeldahl method of protein determination and infra red reflectance measurements in hard red winter wheats. The possible use of biuret method for protein content for mass scale screening and dye binding for basic amino acids was explored by Lein *et al.*[11] Greenway[12] recommended comparative testing of protein methods at five years interval to observe differences which might have developed due to the introduction of new varieties into commercial channels. In this communication we report a comparison of Kjeldahl, dye binding and Grain Quality Analyzer methods for protein content.

Two hundred and seventeen samples of wheat strains and varieties, supplied by the Economic Botanist (Wheat), Haryana Agricultural University, Hissar, and five supplied by Tecator USA were ground to pass through 80 mesh sieve with Udy cyclone sample mill and stored in air tight plastic containers. The moisure content of the samples was determined by AOAC method[13]. For the estimation of Kjeldahl protein the method of McKenzie and Wallace[1] was employed by digesting 500 mg sample with 15 ml sulphuric acid in the presence of potassium sulphate and copper sulphate. Distillation of ammonia was done in Markham nitrogen distillation apparatus and the ammonia so evolved was absorbed in 4 per cent boric acid and titrated against N/100 potassium biiodate. The DBC protein was determined by reacting 800 mg sample with 40 ml dye reagent (1.32 g/l.) for 30 min in Reactor-R shaker at 25 to 30°C. The protein content was calculated by using regression equation (P per cent = 27.40 (1.052-C) after determining the concentration of unbound dye by Udy colour analyzer. The infrared light reflectance was measured using a Neotec Grain Quality Analyzer model GQA 31 EL. The instrument was precalibrated for wheat protein and the well mixed sample was placed to overflowing in the Neotec sample cup and the meal was levelled with a spatula and placed into the spring loaded compressor and tightened. The entire cup was cleaned from outside and then placed in the loader drawer and the drawer closed. Readings for protein percentage were read directly from the instrument. The results are expressed on dry matter basis.

The protein values as obtained by the Kjeldahl, dye binding and Grain Quality Analyzer methods are detailed in Table 1. The moisture content of samples varied from 9.1 to 13.5 percent whereas the average was 10.4 percent. The Kjeldahl protein ranged from 10.8 to 19.4 percent, the protein values by dye binding varied from 8.2 to 16.2 percent and by Grain Quality Analyzer from 9.0 to 18.4 percent. The dye binding capacity method and Grain Quality Analyzer methods have been found superior to Kjeldahl method because of speed of analysis and both the methods measure true protein. Protein

TABLE 1. A COMPARATIVE DATA ON PROTEIN CONTENTS OF WHEAT OF THE THREE METHODS

Kjeldahl*	No. of samples	Av. protein %		
		Kjeldahl	DBC	Grain Quality Analyzer
10.0 – 10.9	4	10.6	11.0	10.7
11.0 – 11.9	33	11.6	11.1	11.2
12.0 – 12.9	59	12.5	11.2	11.9
13.0 – 13.9	51	13.5	12.0	12.4
14.0 – 14.9	43	14.4	12.7	12.9
15.0 – 15.9	17	15.2	13.7	13.8
16.0 – 16.9	10	16.3	14.4	14.5
17.0 – 17.9	3	17.5	15.3	15.9
18.0 – 18.9	1	18.0	16.2	15.9
19.0 – 19.0	1	19.4	14.5	18.4
10.0 – 19.9	222	13.4	12.1	12.4
SE		0.099	0.097	0.083

*N×5.7

estimated by DBC method gives a value which reflects nutritional quality of protein as the dye binds the essential amino acids namely histidine, arginine and lysine.

The Infra-red reflectance instrument also provides means of estimating other constituents such as moisture, oil content, etc. After calibration of the instrument, the operation of the instrument is relatively simple with digital display of results. We have found that grinding of the sample is a critical factor in the case of dye binding and in the Grain Quality Analyzer methods, because accuracy depends upon the dye bound by the particles and light reflectance off the sample surface respectively. Another important feature of the Infra red reflectance instrument is that at no time the samples have to be weighed and after a warm up time (4 hr) it takes only fifteen seconds to measure the protein content. Thus speed of analysis can be considerably enhanced. The correlation coefficients obtained for each of the methods showed significant and positive values. The magnitude of the correlation coefficient between Kjeldahl and DBC methods was ($r=0.734$), between DBC and Grain Quality Analyzer was ($r=0.739$) and between Kjeldahl and Grain Quality Analyzer was ($r=0.999$).

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DETECTION OF PENICILLIC ACID IN FOODS

Two new colour reagents have been developed for visualising penicillic acid on thin layer chromatographic plates. *p*-Tolualdehyde reagent gives a phlox purple colour and *p*-dimethylaminobenzaldehyde reagent gives a chrome yellow colour. The presence of as low as 0.25 μg of penicillic acid could be detected.

Penicillic acid is a well known mycotoxin produced in large amounts by certain species of the Penicillium viz. *P. puberulum*, *P. martensii* and *P. cyclopium*[1]. Various strains of *A. ochraceous* group viz., *A. ochraceous*, *A. sclerotionum*, *A. alliaceus*, *A. ostionum*, *A. melleus* and *A. sulphureus* were also found to produce penicillic acid along with ochratoxin in cereal grain[2]. Conditions for the production of penicillic acid by the *Penicillium* species on different agricultural commodities have been examined by Ceigler and Kurtzman[3]. They have also reported that large quantities of penicillic acid are produced when high moisture corn was stored at low temperatures[4].

A flurodensitometric assay has been described for penicillic acid. The method depends on the conversion of penicillic acid on thin-layer chromatography (TLC) plate by ammonia fumes to a blue fluorescent derivative[5]. The reactions of various toxins on TLC plates with p-anisaldehyde have reported. Penicillic acid is reported to give a green spot under visible light and blue spot under long wave UV-light[6].

During our preliminary screening work on the survey of different food grains for various mycotoxins, we had considerable difficulty in visualising penicillic acid by these two procedures, as many blue spots and streaks were seen with crude extracts. Further, the p-anisaldehyde reaction could not identify penicillic acid since the intensity of the colours developed was low. Consequently it became necessary to develop alternate procedures specific for penicillic acid. We found that *p*-tolualdehyde (TA) and *p*-dimethylaminobenzaldehyde (DABA) give brightly coloured spots with penicillic acid under visible and long wave UV light and the results are reported here.

Pure crystalline penicillic acid was obtained from N.R. R.L., U.S.A. Standard TLC procedures were followed using glass plates coated with Silica Gel G with a thickness of 0.25 mm. The plates were activated at 110°C for one hour and were kept in a desiccator cabinet until used. Solvent used for development of the plates (15 cm) was a mixture of toluene-ethyl acetate-90 per cent formic acid (6:3:1). The aldehyde spray reagents were prepared with the following composition: TA or DABA-concentrated sulphuric acid-acetic acid-methanol, 1:5:10:70.

Penicillic acid was dissolved in chloroform and spotted at various concentrations between one and five μg. After the chromatography the plates were allowed to dry and the colour reagents were sprayed. After spraying, the plates were heated and maintained at 130°C for 5 to 8 min when distinct bright coloured spots developed. However, before heating no visible colour could be seen. The colours were compared with Horticultural colour charts[7]. With TA the spots appeared phloxpurple 632/2* under visible and under UV light. The colour was stable for 15 min and then it slowly changed towards yellow and faded off from visible light within 6 hr. But under UV light although the colour slowly faded, it was noticeable for 24 hr. Different concentrations of penicillic acid were tested and quantities as low as 1.0 μg could be detected and 2.5 μg gave bright spots.

With DABA the colour developed was chrome yellow 605/2 under visible light and primrose yellow 601/1 under long wave UV light. Quantities as low as 0.25 μg of penicillic acid could be detected while 1.25 μg gave a bright spot. The colour was stable for 30 min. after which it started turning brown, and in 7 hr the colour faded considerably, both in visible and UV light.

With both the spray reagents, the colour, could again be visualized by reheating the plates briefly at 130°C and the colour of the spots intensified with the increase of the concentration of the toxin. So also the colour intensified with the increase in concentration of the aldehyde in both the spray reagents and we found that 1.0 ml (TA) or 1.0 g (DABA) of the aldehyde gave the best results.

The extraction of the coloured spots from the TLC plates was not possible with solvents like water, ethyl alcohol, chloroform, hexane, acid and alkali in the case of TA reagent. However in the case of DABA reagent, extraction of the spot with ethyl alcohol resulted in a pink coloured solution. This was verified by spraying the TLC plates with ethanol. This resulted in pink spots which could be used as a further confirmatory test.

But, we were not able to detect penicillic acid as a natural contaminant in detectable amounts, eventhough we have examined over thousand food grain samples. So the application of this to food grains containing naturally occurring penicillic acid, derived due to fungal contamination could not be tested. It is, therefore, possible that the fungi which had contaminated the wheat and rice had not produced penicillic acid in appreciable amounts.

*The numerator indicates the hue of the color and the denominator indicates the intensity of that particular hue. As the numerical value of the denominator increases the intensity of the hue decreases.

However, the above results were verified using samples of wheat and rice to which known amounts of penicillic acid were added. The samples were extracted with chloroform and the extract was concentrated under vacuum and then spotted directly on TLC plates. In all cases easy identification of penicillic acid was possible. We hope that these two new spray reagents for penicillic acid will be found useful for its detection in various foods.

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STANDARDISATION OF EXTRACTION OF PUNGENCY FROM WHOLE CHILLI (CAPSICUM) FOR ESTIMATION OF CAPSAICIN

Capsaicin was extracted from chilli (capsicum) powder using 6 different types of solvents. The per cent capsaicin extracted by these solvents ranged from 0.13 to 0.22%. Acetone was selected among these as it gave highest extraction (0.22%) of capsaicin. Optimum period of extraction was found to be 3 hr. Capsaicin content of 14 varieties of chilli was determined.

Literature on the estimation of capsaicin content in chilli has been recently reviewed.[1,2] It is found that in the methods described, different solvents were used for

TABLE 1. EXTRACTION OF CAPSAICIN FROM CHILLI POWDER, USING DIFFERENT SOLVENTS

Solvent	% Capsaicin
Ethylene dichloride	0.13
Methylene chloride	0.14
Chloroform	0.18
Methanol	0.20
Acetone	0.22
Ethanol (95%)	0.22

Extraction time 2 hr; Wt. of chilli powder: 10 g.

TABLE 2. EXTRACTION OF CHILLI POWDER WITH ACETONE FOR DIFFERENT PERIODS

Extraction	% capsaicin
1	0.22
2	0.23
3	0.25
4	0.25
10	0.25

TABLE 3. ANALYSIS OF CHILLI VARIETIES FOR CAPSAICIN CONTENT

Variety	% capsaicin
Kandu	0.22
Kayari Gudda	0.32
Gatlong	0.42
Kansar	0.37
Jurg	0.42
Chandni I	0.35
Chandni II	0.41
Darable	0.35
Tikri	0.43
Katwar	0.12
Timbi	0.28
600/4-2-3-6	0.37
419/1-4-4-3	0.18
Jwala	0.53

extraction and these methods have not so far been compared. Hence an experiment was undertaken to select a most suitable solvent for extraction of capsaicin and to standardise the method of extraction from chilli.

The solvents used in the study included chloroform, methylene chloride, ethylene dichloride, methanol, acetone, and ethanol (95 per cent). Chilli was powdered to pass through 30 mesh sieve and 10 g of this powder was used; Soxhlet extraction was done for 2 hr. From the extract, capsaicin was estimated by spotting on TLC silica gel plate as per the method of Mathew *et al*[3].

The results are given in Table 1. The capsaicin extracted ranged from 0.13 to 0.22 per cent depending upon the solvent used. Acetone and ethanol (95 per cent) extracted a maximum of 0.22 per cent capsaicin from the chilli powder. Acetone was selected for further studies as it has a lower boiling point. To determine the optimum time, the extraction was carried out for 1, 2, 3, 4 and 10 hr with 10 g of chilli powder and the results are given in Table 2. It can be seen, that the yield of capsaicin extracted increased up to 3 hr (0.25 per cent) beyond which it remained constant. Hence 3 hr was taken as optimum for extraction purposes.

Capsaicin was estimated in 14 chilli varieties (11 varieties obtained from CFTRI, Mysore and 3 varieties from University of Agricultural Science, Bangalore), using acetone as solvent and extracting for 3 hr. The values are given in Table 3. The capsaicin content in these 14 chilli varieties ranged from 0.12 to 0.53 per cent.

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SELECTION OF FLEXIBLE SINGLE-SERVICE, CONTAINERS FOR IN-PACK PROCESSING OF MARKET MILK

An effort was made to find out the possibility of using flexible single service containers for in-pack processing of milk. Preliminary screening of the pouches for heat tolerance was done by immersing in boiling water bath. Further heat tolerance test was conducted with 6 pouches by subjecting to temperatures ranging from 75°C to 90°C and in boiling water for 10 to 12 min. Flavour imparting property was tested by in-pack processing of milk at 75 and 85°C for 5 min. and evaluating consumers opinion for leakage, handling container appeal etc. It is concluded that Mylar polyethylene and cellophane laminating were found most suitable for in-pack processing. From consumers evaluation the cellophane laminated pouch scored maximum.

In India today there is a growing awareness of the mertis of hermatically packed milk and other dairy products. Flexible single-service containers are playing

an important role these days in food packaging industry. These are not only in use for retail packaging of market milk but are also used for boil—in bag processing of various food products other than market milk. In-pack processing of market milk is being done by heating and cooling of raw milk at various temperature-time combinations after packaging and sealing in single-service flexible pouches. Heating and cooling are done by placing the sealed pouches in a suitable cage and immersing them in hot and cold water.

The high strength of polythylene bags, their lightness, freedom from tampering, non-contamination of the product after sealing, and ease of disposal are some of the reasons for the use of plastic bags in packaging milk[1]. Fluckiger[2] reported that between 1957 and 1962, the proportion of single-service containers for packaging market milk in Switzerland increased from 0.9 to 90 percent.

Over 50 processors are now producing boil-in-bag foods in the U.S.A. where sales for 1965 were expected to reach 350 million bags[3]. Rigidex high density polyethylene, which is tasteless and odourless, is being used by Shorke Metal Box Ltd. for the production of packs for boil-in-bag food[4]. Sacharow[5] reported that the all film-structure used by over 85 per cent of the boil-in-bag industry is generally composed of 0.5 mil polyester and 1.5-2.0 mil polyethylene. It utilizes the strength and inherent barrier properties of polyester and the heat sealable character of polyethylene.

An effort has been made to make use of the available flexible single-service containers for in-pack processing of market milk and for this process it was a prerequisite to select suitable containers based upon various physical and organoleptic characteristics. The plastic pouches for the study were procured from various firms and other organisations within the country and abroad, which are being used for packaging milk and other food products. After preliminary screening of these containers, detailed study of a few was made on the basis of heat tolerance, leakage, heat-sealability and flavour effect. Later on, consumers' acceptance tests were also performed on the selected pouches.

Preliminary screening of the various pouches for heat tolerance was done by filling the packages with water, immersing in boiling water-bath for 10 to 12 min and observing the extent of leakage and bursting of the packages. Samples showing heavy leakages, pin-holes or bursting, were rejected for in-pack processing. For the determination of suitability of the selected pouches for in-pack processing of milk, a detailed study of heat tolerance and flavour imparting property was made on six types of packaging materials after studying the behaviour of various packages.

To determine heat tolerance, the plastic pouches were filled with the coloured water and after sealing were immersed in hot-water at various temperatures. Red coloured water was used for easier detection of leakages. After heating and cooling the packages, the adhering water is wiped off thoroughly with the help of dry cloth and examined for leakages and pin-holes. On the basis of leakages, heat tolerance of pouches was determined at different temperatures. Heat tolerance of six kinds of pouches was studied at 75°, 80°, 85°, 90°C and in boiling water for 10 to 12 min. Three to six packages were processed at each temperature and after cooling with tap water, leakages were detected.

To determine flavour imparting property, raw milk was in-pack processed at 75° and 80°C for 5 min in 5 pouches of each 6 type containers and in glass container as the control. Thus, a total of 35 samples including control samples were processed at each temperature. Five judges scored the plastic flavour imparted to the

TABLE 1. PHYSICAL AND ORGANOLEPTIC CHARACTERISTICS OF SOME SINGLE-SERVICE PLASTIC CONTAINERS

Kind of pouch	Heat tolerance	Leakage at 70–80°C	Flavour defect of milk	Suitability for in-pack processing of milk
Paper/Aluminium/Polyethlene laminate	Boiling water	—	Pronounced	Unsuitable
Mylar/Polyethlene laminate	,,	Very low	Nil	Suitable for higher temp.
Cellophane/Polyethylene laminate	90°C	,,	,,	Suitable for higher temp.
Polyethylene blackened thin (300 gauge)	85°C	Low	,,	Suitable for lower temp.
Polyethylene translucent thin (300 gauge)	80°C	,,	,,	Suitable for lower temp.
Polyethylene translucent thick (800 gauge)	80°C	Heavy	Slight	Unsuitable

All the containers except the first one in the above list are readily sealable.

TABLE 2. SUITABILITY OF SELECTED POUCHES FOR IN-PACK PROCESSING OF MARKET MILK BASED ON SENSORY EVALUATION TESTS

Type of container	Numerical scores (calculated) from frequency to response				Total	Remark
	Leakage	Abnormal flavour	Easiness in handling	Container appeal		
Mylar/Polyethylene laminate	-9	-18	273	249	495	II
Polyethylene Blackened thin	-9	-9	201	285	468	III
Cellophane/Polyethylene laminate	-9	-9	297	294	573	I
Polyethylene Translucent thin (300 gauge)	-18	-18	177	210	351	IV

milk. Judges were previously made familiar with the plastic flavour imparted from an ordinary plastic container to milk.

On the basis of the above preliminary tests three laminated and three non-laminated polyethylene pouches were used for detailed study. Physical and organoleptic characteristics of plastic pouches studied for use in in-pack processing of milk are presented in Table 1.

The paper/aluminium/polyethylene laminate tolerated heat upto boiling point of water, but its polyethylene imparted pronounced plastic flavour to milk and due to slow heat penetration through paper and aluminium, it took more time to seal, and was, therefore, rejected for in-pack processing. Mylar/polyethylene and cellophane laminates were found most suitable for in-pack processing of milk, as these were tolerant to heat at 90°C, inert to milk, easily heat sealable and with minimum leakages during in-pack processing. Sacharow[5] also reported the maximum use of films composed of polyester and polyethylene in boil-in-bag industry. Thin black polyethylene and translucent polyethylene were found moderately suitable for in-pack processing of milk.

The four containers which were mainly used in the experiments, were further ranked on the basis of consumer's acceptance test. Every container was used for the supply of in-pack processed milk (75°C for 5 min) to 45 consumers in their homes for two days. Thus a total of 360 packages of 250 ml capacity each were supplied. These consumers were asked to give their remarks for leakage, flavour, easiness in handling the pack and container appeal on each kind of pouch. For convenience of grading, the observations have been converted into numerical scores. Each remark is given score out of 9. For leakage or abnormal flavour remark, total score of 9 were deducted, while easiness in handling and container appeal were divided into poor, fair, good and excellent categories and given scores of 0, 3, 6 and 9 each, respectively. The brief tabulation of the score is presented in Table 2.

As will be seen from the data, the cellophane laminated pouch scored maximum, probably because of its tough body and attractive shape to enable it to remain standing like a bottle. Thin translucent polyethylene scored lowest probably because of its loose body and less attractive characteristics than others. Moreover, the leakages detected by the consumers were also comparatively more in this case.

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CARBOFURAN RESIDUES IN POTATOES

Carbofuran residues were estimated in potato tubers when various doses of the insecticide were applied to the soil at the time of planting. The residue ranged from 0.047 to 0.295 ppm, in the different treatments, the levels increasing with increase in dosage. At the highest dose of 44.5 kg. Carbofuran 3G per acre, the residue in the tuber was less than the limit of WHO of 0.5 ppm. Cooking the tubers further reduced the residue levels by 50 to 90 per cent.

Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzo-furanyl -N-methyl carbamate), is a broad spectrum systemic insecticide and neamaticide used extensively against various crop pests for soil application[1]. Potato is an important crop on which encapsulated Furadan* 3G

*Furadan is the registered trade mark of FMC Corporation for carbofuran.

TABLE 1. CARBOFURAN RESIUDES IN POTATO TUBERS

Place of trial	Variety	Carbofuran 3G dose (kg/acre)	Method/time of application	Interval between application and harvest (days)	Carbofuran residues (ppm) Raw tubers	Cooked tubers
Ooty (Finger post)	Holland	15	Planting	129	0.080	—
		25	,,	129	0.191	0.010
Ooty (Anandagiri)	,,	15	,,	123	0.095	—
		25	,,	123	0.170	—
		35	,,	123	0.190	—
		Tubers dipped in 0.05% carbofuran for 3 min. before planting		123	0.047	—
		44.5	Planting	123	0.295	0.133
Ooty (Palada)	Kufri Jothi	25	,,	110	0.098	—
		25	,,	110	0.063	0.022

is being ivestigated for the control of insects and golden nematode in the Nilgiris, at dosages ranging from 15 to 44.5 kg per acre, given as spot application close to the tubers at the time of planting. In these trials a new formulation, Furdan 40F, which is a suspensible paste, is also being investigated. In this paper the residue of carbonfuran in potato tubers at the time of harvest is dealt with.

Field experiments were conducted on potato (Var. 'Holland' and 'Kufri Jothi') at different locations in Ooty, applying encapsulated Furadan 3G at rates ranging from 15 to 44.5 kg/acre (1.125 to 3.34 kg a.i./ha) at the time of planting. In one of the treatments, the planting tubers were dippped in 0.05 percent carbofuran suspension for 3 min before planting. The crop was planted during the third week of February 1977 and harvested during the first week of July 1977 The details of the treatments are given in Table 1. In the harvested tubers Carbofuran residue was extracted and the extracts were subjected to clean-up steps as described by Cook[2]. The extracts were then evaporated to almost dryness, dissolved in a small volume of methanol and carbofuran was estimated colorimetrically as described by Gupta and Dewan.[3] The cut tubers were treated with about 1 per cent NaCl and cooked in an autoclave at 0.7 kg Cm^{-2} steam pressure for 5 min and the residue was estimated.

Data presented in Table 1 indicate that the residue of carbofuran increased in the tubers with increase in doses applied. The residue levels ranged from 0.047 to 0.295 ppm in the different treatments. It was lowest in the treatment where the tubers were dipped in 0.05 per cent carbofuran solution and was highest (0.295 ppm) when Furdan 3G was applied @ 44.5 kg/acre. In all cases the residues were well within the tolerance limit of 0.5 ppm fixed by WHO/FAO[7] for potatoes. Although carbofuran is known to persist in the soil with half life periods ranging between 30 and 60 days, in plants the carbamate is much less persistent[4]. Hence, in spite of the high doses of the toxicant applied to potato in this study, its residue in the tubers was low.

In the literature no information is available on the carbofuran residue in potao tubers. However, Rajukkannu *et al*[5] in their studies with sweet potato observed that with the application of 1.0 kg carbofuran a.i. per hectare (i.e 33 kg of 3G) only 0.103 ppm was present in the tubers as residues at the time of harvest. Results of the present study reveal that it is safe to use carbofuran on potato for nematode control even at rates as high as 44.5 kg of 3G, per acre.

The results of cooking study reveal that the residue levels were reduced considerably upon cooking. The extent of reduction varied from 50 to 90 per cent depending upon the dosage used. Processing of vegetables is known to bring down the residue levels of most pesticides[6]. Therefore, it can be concluded that from point of view of residues, Carbofuran is a safe nematicide and insecticide on potato.

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RESIDUES OF DDT AND ENDOSULFAN IN GRAM-GRAINS

Residues of DDT and endosulfan were estimated by using GLC and TLC in the harvested gram, *Cicer arietinum* var. *Kabli* obtained from the crop sprayed with DDT and endosulfan at the dosage of 0.5 and 0.1 kg a.i./ha respectively at 56 days before harvest. DDT residues were present mainly as p,p' — DDT with traces of p,p' — DDE at a mean level of 0.068 ppm. Endosulfan residues which occured as its isomers and diol were found to be 0.035 ppm.

DDT and endosulfan have been reported to be effective for the protection of gram (*Cicer arietinum* var *Kabli*) crop against the attack of gram caterpillar (*Helicoverpa armigera* (Hubner)[1,2]. The application of these insecticides is recommended at the pod formation stage which is liable to result in the contamination of kernels inside the pods. The present study was, therefore, carried out to assess the extent of residues resulting from such a recommended usage of these insecticides.

Kabli gram, variety "L 550", was sown on the 18th October, 1974 in plot size of 5×4 metres as irrigated crop. DDT 50 per cent wettable powder (WP) and Endosulfan 35 per cent emulsifiable concentrate (EC) were sprayed at 0.25 per cent and 0.05 per cent concentrations respectively using 200 l. of water/acre on the 25th Feb., 1975. Water alone was sprayed on simultaneously maintained control plots. There were 2 replications for each of the treatments.

About one kg sample of the gram pods were collected from each of the treatments at harvest on the 23rd April, 1975. The dried grains were ground and extracted with acetonitrile-water (65:35, v/v) by overnight immersion.[3] The acetonitrile extracts after dilution with 500 ml 2 per cent aqueous sodium chloride solution were partitioned into petroleum ether. The petroleum ether extracts were concentrated and further cleaned by column chromatography using activated Florisil for DDT[4,5] and activated neutral alumina for Endosulfan[6]. Analyses were done by gas-liquid chromatography and thin-layer chromatography as described by Dhaliwal and Kalra[7].

Recoveries of p,p'-DDT and p,p'-DDE from fortified samples were 87 and 92 per cent respectively. Similarly recovery of endosulfan A and B were 90 and 92 per cent respectively. The minimum limits of estimation of DDT and DDE were 0.05 ppm while that of endosulfan A and B were 0.01 and 0.02 ppm.

The results obtained indicated the presence of DDT and Endosulfan in the gram grains at harvest (Table 1). Since the gram grains were located inside the pods at the time of spray, it is quite probable that these insecticides had penetrated into the grains where these peristed till harvest. However, the quantities found were very small. DDT residues were found mainly in the form of p,p'-DDT (0.068 ppm) while only traces of p,p'-DDE were detected. No other principal metabolite or isomer of DDT could be detected. Endosulfan residues were present as endosulfan A and B, the latter being more than the former though traces of endosulfan diol were also present. The occurrence of low residues of DDT and DDE (0.05 ppm) in pulses imported into Britain,[8,9] also supported the possibility of contamination through the use of DDT in this manner.

TABLE 1. DDT AND ENDOSULFAN RESIDUES IN GRAM-GRAINS AT HARVEST

Replicates	Residues in ppm		
	p,p'-DDT	Endosulfan A	Endosulfan B
I	0.075	0.01	0.03
II	0.050	0.01	0.02
Mean	0.068	0.01	0.025

The residues of p,p'-DDE and endosulfan diol were present in traces.

The tolerance limits of these insecticides on gram and other pulse crops are not available either at the national or international level. However, the residues of both the insecticides encountered were quite low. The recommended usage of DDT and endosulfan on gram crop, therefore, does not seem to constitute any residue hazards.

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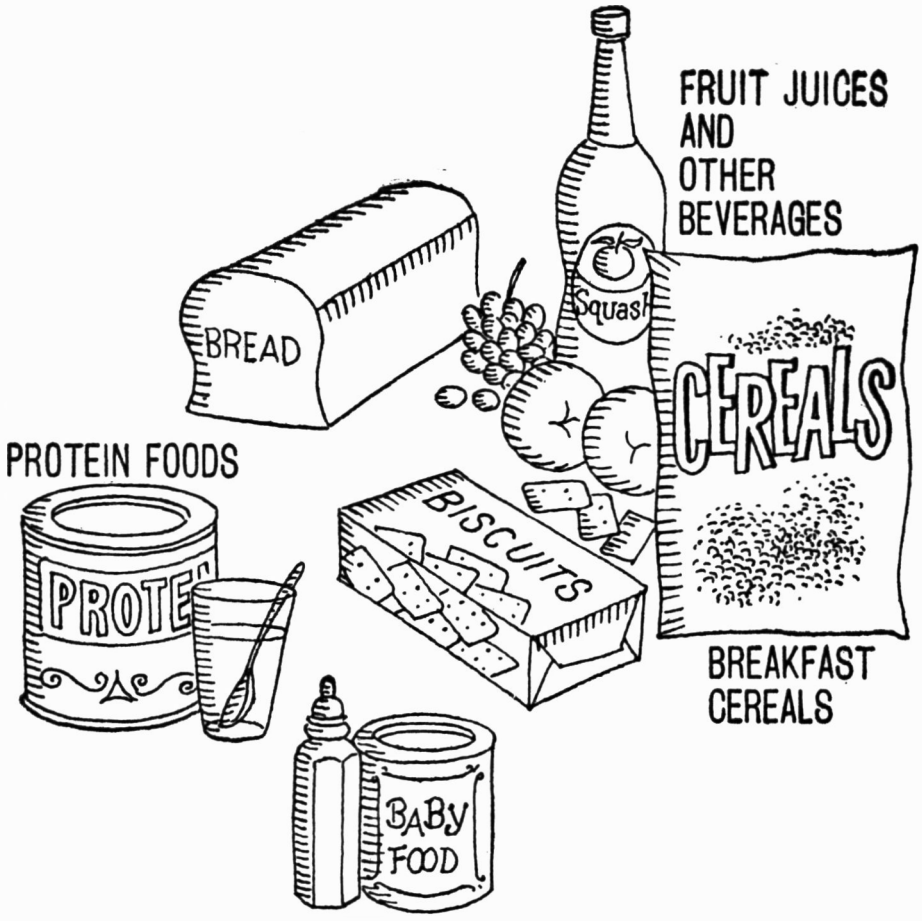

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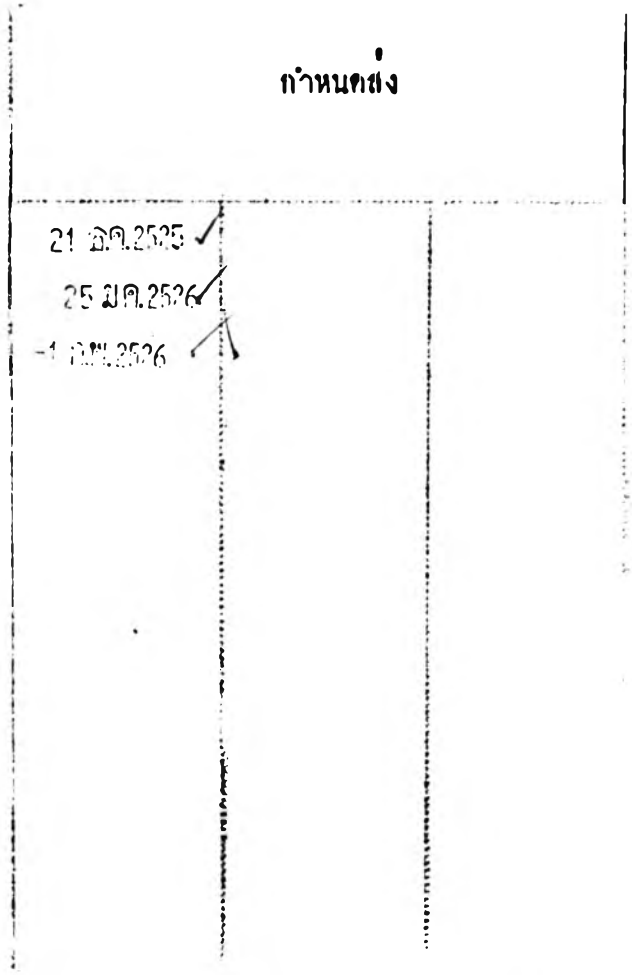
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