COMMUNICATIONS

Nonenzymatic Discoloration in Dried Cabbage. Ascorbic Acid—Amino Acid Interactions

Dehydroascorbic acid (DHA) and 2,3-diketogulonic acid (DKG), formed from ascorbic acid (AA) during dehydration of cabbage, react nonenzymatically with the free amino acids to cause red to brown discoloration in the dried product. Using model systems, the mechanism of color formation has been shown to involve the Strecker degradation of amino acids to aldehydes. An alcoholic medium and AA instead of preformed DHA or DKG are more favorable for aldehyde formation at a low temperature (40° C.). The red color formed initially in freeze-dried cabbage and in model systems has a pH optimum in the range of 5.3 to 6.5 for development and behaves like the rearranged product of a Schiff base. The conversion of red color to brown is accompanied by the liberation of carbonyl groups. Blanching causes oxidation of AA and enhances the extent of browning in the hot air–dried product.

Investigations on the nature of leucoanthocyanins in cabbage (Ranganna and Govindarajan, 1966) revealed that freeze-dried cabbage developed a light cherry-red color at the end of drying or on storage for a few days which gradually faded and changed to a brown color on prolonged storage. An alcoholic extract of fresh cabbage gave similar color changes. This discoloration has now been shown to result from the reaction between AA and the free amino acids present in cabbage. The mechanism of amino acid–AA interactions has been studied in model systems. The effect of different drying conditions of cabbage has also been investigated to elucidate the chemical reactions involved.

Hodge (1953) and Reynolds (1963, 1965) have reviewed the extensive literature on nonenzymatic discolorations. Although AA-induced browning in citrus products, which involves its conversion to furfural, is well known (Braverman, 1963), discoloration involving the reaction of amino acids with DHA or DKG has not been widely investigated in other foods.

EXPERIMENTAL

Cabbage from a local market was utilized in these studies. After washing, the inner white to pale green leaves were used. Unless otherwise stated, commercial absolute alcohol was employed. Color was measured with an Eel colorimeter using the 624 filter (520 mμ). Absorption spectra were taken in a Beckman DU spectrophotometer.

Preliminary Observations. Shredded cabbage, freeze-dried with or without blanching, developed a light red color which was less pronounced in the former. The color which also formed in alcoholic extracts of the cabbage was nonenzymatic in nature and developed only in polar solvents such as ethanol, methanol, and acetone. Aldehydes or other impurities present in commercial alcohol were not responsible for this color change. AA, when added to the alcoholic extract of cabbage, considerably enhanced the red color. The rate of formation of color increased with an increase in storage temperature.

Isolation and Identification of Reactants with AA in Color Formation. Minced cabbage (5 kg.) was shaken twice with alcohol (7.5 and 5.0 liters) for 1 hour, filtered, the filtrate shaken twice with Pattinson's calcined magnesia (5.0 and 2.5% w./v.), and again filtered. The adsorbent was mixed with sufficient water to make a thin paste, shaken for 1 hour, and filtered. The filtrate (3.4 liters) was treated with activated carbon (85 grams) to remove the yellow color and was freeze-dried. A yellow hygroscopic powder (15.33 grams) was obtained which was dissolved in water and mixed with twice its volume of alcohol. A white precipitate formed. A second, third, and fourth crop of precipitate was obtained by successive additions of alcohol. These fractions were free from thiocyanate, *o*-dihydric phenols, and leucoanthocyanins (Mark *et al.*, 1959; Peltzer, 1953; Ranganna and Govindarajan, 1966) which have been implicated in the pink discoloration of pickled or processed cabbage. The first fraction contained only sulfur compounds and did not develop a red color with AA in 70% alcohol, while the remaining fractions, which contained ninhydrin-positive compounds, developed color. Amino acids present in the isolate were identified by the buffered paper chromatographic method of McFarren (1951).

Amino Acid–AA Model Systems. In separate test tubes, 20 mg. of each of the amino acids was mixed with 30 mg. of AA in a final volume of 15 ml. with alcoholic strengths ranging from 20 to 100%. An aqueous mixture served as control. The test tubes were corked, and the color was measured at frequent intervals of storage at room temperature (24° to 27° C.).

Reaction of Amino Acids with DHA and DKG. AA was oxidized to DHA with iodine in an alcoholic solution and to DKG by treatment with bromine (Association of Vitamin Chemists, 1966). The pH of 0.01*M* solutions of DHA and DKG in 80% alcohol was around 2.0. These solutions did not develop the red color with glycine. The pH of glycine-AA model systems was around 4.9, and the color developed without any further pH adjustment. To study the optimum pH for color development in glycine–DHA/DKG system, 1.0 ml. of 0.02*M* glycine solution was mixed with 2.0 ml. of a 0.01*M* solution of DHA or DKG, the pH was adjusted to various levels (using 4% sodium hydroxide or citric acid in 80% alcohol) and the volume made up to 20 ml. with 80% alcohol. The mixture was stored at 37° C. and the color measured until it reached a maximum. The optimum pH for color development in the glycine–DHA system was 5.8 to 6.5 and in the glycine–DKG system, 5.3 (Figure 1). No red color developed below pH 3.5.

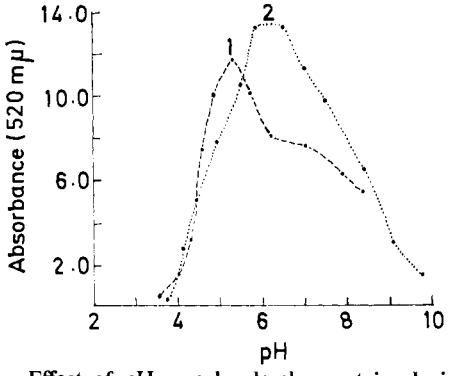

Figure 1. Effect of pH on color development in glycine-DHA and glycine-DKG systems

1. Gly-DKG 2. Gly-DHA

To study the color reaction of different amino acids with DHA and DKG, 0.01*M* solutions of these in 80% alcohol were prepared, and the pH was adjusted to the optimum range. Five milliliters of the amino acid solution were mixed with 5.0 ml. of DHA or DKG and 10.0 ml. of 80% alcohol, and the color was measured at frequent intervals of storage at room temperature.

The color reaction in an aqueous medium was studied by the method of Koppanyi *et al.* (1945).

Analysis of Products of Reaction between Amino Acid and AA/DHA/DKG. The apparatus used in these estimations is shown in Figure 2. A regulator-controlled mantle was used for heating. The reaction conditions are given in Table I. Aldehyde-free alcohol used in these studies was prepared by the A.O.A.C. method (1965). The pH of the reaction mixture was adjusted to 5.6. Formaldehyde was detected by using chromotropic acid (Boyd and Logan, 1942) and acetaldehyde from its characteristic smell and the melting point of its 2,4-dinitrophenylhydrazine (2,4-DNPH) derivative (Schönberg *et al.*, 1948). When the reaction was carried out overnight or longer, the aldehyde produced was absorbed in a 1% 2,4-DNPH solution in alcohol and estimated by a modification of the 2,4-DNPH method (Critchfield, 1963). Aldehyde-free ethanol was used in place of methanol, 10 ml. of alcohol and 1 ml. of water instead of 80% pyridine, and 16.5% instead of 33% potassium hydroxide.

The carbon dioxide evolved from the reaction mixture was swept by carrier gas (CO_2-free air or N_2) into an alkali

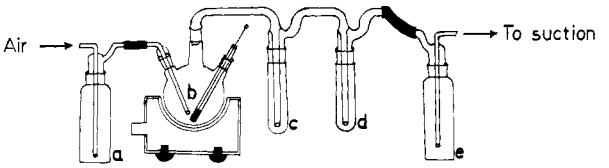

Figure 2. Apparatus for the determination of aldehyde and carbon dioxide

a. NaOH solution *b.* Reaction mixture *c.* Water or 2,4-DNPH solution *d.* Standard NaOH solution *e.* NaOH solution

trap containing 1*N* NaOH, and estimated by the method of Lalikainen *et al.* (1958).

Qualitative Test for Schiff Bases. The red color from model systems and from freeze-dried cabbage was chromatographed using 1-butanol–water–ethanol (20:5:1.3) in a descending direction for 36 hours. The color, which remained near the base line, was rechromatographed and allowed to air-dry in the dark. The rearranged products of Schiff bases of DHA or DKG were traced by testing for the free $-NH_2$ group and DHA or DKG moiety.

Changes during Drying. Cabbage was washed, the outer green leaves removed, and cut into shreds. One lot of shreds was dried without any pretreatment. The second lot was blanched in steam for 5 minutes, allowed to cool, and dried. In the third lot, blanched cabbage was sulfited in 0.2% potassium metabisulfite solution for 15 minutes, drained, and then dried. Drying was done in a Stokes freeze-dryer without application of heat at any stage and in a cross-flow electrically heated hot air (60° C.) cabinet dryer having a truck for mounting the trays containing the prepared material.

AA, DHA, and DKG were estimated by the method of the Association of Vitamin Chemists (1966). To estimate the amino acids in fresh as well as dried cabbage, the latter was homogenized in alcohol with water added wherever necessary to adjust the final concentration of alcohol to 70% and centrifuged. The supernatant was concentrated in a vacuum desiccator and chromatographed three times in a descending direction using 1-butanol–water–ethanol (20:5:1.3). The red color in freeze-dried cabbage and the brown color in hot air-dried cabbage containing the reacted amino acids remained on the base line, while the unreacted amino acids separated. The latter were estimated by the method of Giri *et al.* (1952). For measurement of the extent of browning, 10 grams of

Table I. Determination of Aldehyde and Carbon Dioxide Formed in the Amino Acid–AA Reaction

Concentration, Millimoles		Reaction Conditions					Yield, Millimoles	
		Medium	Vol. of reaction mixture, ml.	Carrier gas	Temperature, ° C.	Time, hours	Aldehyde	CO_2
Glycine	50 + DHA 50	A[a]	200	CO_2	80[c]	0.5	1.77	...
Glycine	50 + DKG 50	A	200	CO_2	80[c]	0.5	1.60	...
Glycine	25 + AA 100	A	200	CO_2-free air	80[c]	24	5.60	71.20
Glycine	25 + AA 100	W[b]	200	CO_2-free air	80[c]	24	0.53	65.39
Glycine	25 + DKG 100	A	200	CO_2-free air	80[c]	24	4.34	74.98
Glycine	25 + DKG 100	W	200	CO_2-free air	80[c]	24	0.59	79.56
Glycine	50 + DKG 50	A	400	N_2	55 ± 2[c]	96	3.01	46.59
Glycine	50 + DKG 50	A	400	N_2	37 ± 2	96	1.65	39.83
Glycine	12.5 + AA 50[d]	A	400	CO_2-free air	40 ± 1	168	6.20	39.71
Alanine	12.5 + AA 50[d]	A	400	CO_2-free air	40 ± 1	168	6.63	34.64

[a] A = 70% alcohol. [b] W = water. [c] Reaction carried out in the apparatus shown in Figure 1 using the condenser in addition. [d] 50 ml. of alcohol added to the reaction mixture after 3 or 4 days of reaction to maintain the volume.

sample were blended with 300 ml. of water. To 10 ml. of the filtrate, 20 ml. of alcohol were added and the color was measured at 440 mμ in a Spectronic 20 colorimeter/spectrophotometer.

RESULTS

Amino Acid–AA/DHA/DKG Model Systems. The calcined magnesia isolate from cabbage contained 16 free amino acids: alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, histidine, isoleucine, lysine, methionine, proline, serine, threonine, tyrosine, tryptophan, and valine. In the model systems, all of these amino acids except proline, and in addition leucine and phenylalanine, gave similar color changes with AA in an alcoholic medium. The red color did not develop when the alcohol concentration was below 60%, and the intensity increased with an increase in the alcohol concentration. The color did not develop in the aqueous medium. During storage, the red color gradually faded and changed to brown. The pattern of color change with all of these amino acids was similar to that observed in the lysine–AA system (Figure 3). The stored alcoholic extract of cabbage behaved similarly. Proline (and also hydroxyproline) developed a chrome yellow color initially which turned dark brown on prolonged storage. A continuous increase was noted in the absorbance of the color.

When some of the model systems were examined after storage for residual amino acids, a drop in the amino acid content was observed. The loss was greater at higher alcohol concentrations (60 to 90%) than in an aqueous medium or at lower concentrations of alcohol. None of the systems contained residual AA.

The absorption maximum of the red color was between 510 and 515 mμ, except in the case of aspartic acid (512 to 520 mμ). The red color from the alcoholic extract of fresh or freeze-dried cabbage, and from the reaction mixture containing calcined magnesia isolate and AA, absorbed almost in the same region (514 mμ) (Figure 4). Loss in red color in the above systems was accompanied by the disappearance of the 510- to 515-mμ peak and a concurrent

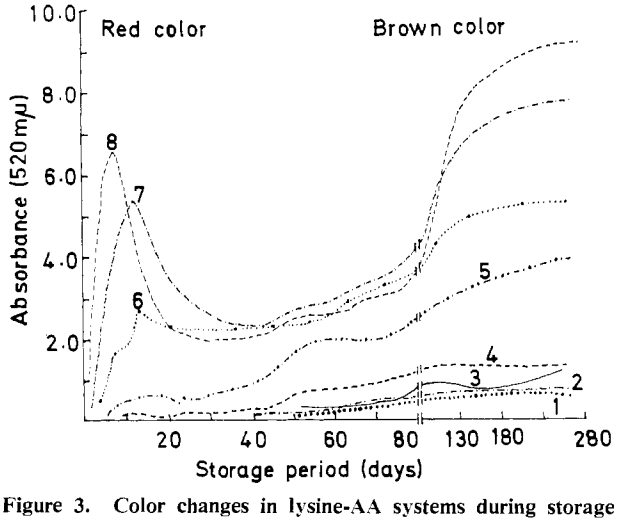

Figure 3. Color changes in lysine-AA systems during storage

1. Water 2. 18.6% alcohol 3. 37.2% alcohol 4. 55.7% alcohol 5. 65.2% alcohol 6. 74.5% alcohol 7. 83.8% alcohol 8. 93.1% alcohol

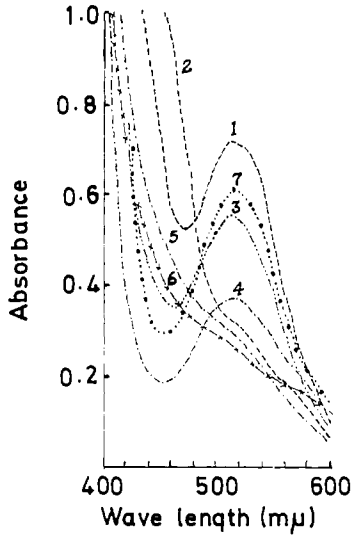

Figure 4. Absorption spectra of red color from lysine-AA model system and cabbage

1. Lysine-AA model system
2. After fading of red color in 1 during storage
3. Red color from alcoholic extract of cabbage
4. Chromatographically separated red color from freeze-dried (unblanched) cabbage
5. After fading of red color in 4 during storage
6. Chromatographically separated color from hot air-dried cabbage
7. Red color from calcined magnesia isolate of cabbage and AA in 70% alcohol

increase between 400 and 440 mμ. Color obtained with proline and hydroxyproline showed no peak between 400 and 700 mμ. Under similar experimental conditions on prolonged storage, the intensity of brown color produced by these two amino acids was far greater than that from the other amino acids.

The amino acids reacted with DHA at pH 5.3 and DKG at pH 5.8 as in the amino acid–AA system. For the same amino acid, the intensity of color was greater with DHA than with DKG.

In the aqueous medium, the red color was produced only at high concentrations of the reactants, and the intensity of the color produced was, furthermore, very low (Table II).

Products of Amino Acid and AA/DHA/DKG Reaction. Strecker degradation of amino acids by DHA at high temperatures (refluxing condition) has been shown in aqueous (Schönberg *et al.*, 1948) and methanolic (Pecherer, 1951) media. At low temperatures (37° or 55° C.) using AA and labeled glycine in an aqueous medium (pH 3.7 or 7.0) with oxygen as the carrier gas, Lalikainen *et al.* (1958) could detect neither Schiff bases nor aldehydes. From a comparative study of the conditions employed, the use of AA instead of preformed DHA or DKG and an alcoholic medium (70%) with CO_2-free air as the carrier gas were favorable for aldehyde formation from amino acids at a low temperature (40° C.) (Table I). Even at high temperatures, an alcoholic medium was preferable

to the aqueous one. Glycine produced formaldehyde, and alanine produced acetaldehyde. In Strecker degradation, for every mole of amino acid which reacts, one mole each of aldehyde and carbon dioxide are produced. Carbon dioxide produced was far in excess of the aldehyde (Table I), which may be due to multiple decarboxylation of AA (Jackson *et al.*, 1960). When DKG was used instead of AA, even with nitrogen as the carrier gas, most of the decarboxylation occurred in the first few hours of the reaction at 55° C. and within 24 hours at 37° C., which can be attributed to the instability of DKG (Euler and Hasselquist, 1952, 1953).

The red color on the chromatograms gave a positive test with ninhydrin indicating the presence of a free $-NH_2$ group. When 2% 2,4-DNPH in $9N$ H_2SO_4 (Association of Vitamin Chemists, 1966) was added to the metaphosphoric acid (5%) extract of red color, osazone formation was observed after incubation overnight at 37° C., thus indicating the presence of a >C=O group, possibly of modified DHA or DKG.

Changes in Red Color during Storage. Chromatographically purified red colored solutions from model systems or freeze-dried cabbage did not have a peak in the ultraviolet region. The red color, when taken up in water, became colorless after storage at 37° C. for 2 or 3 days, and a precipitate formed. This was centrifuged, and the supernatant extracted with ether. The supernatant had an absorption maximum between 266 and 267 mμ (in water) and gave an immediate precipitate with 2,4-DNPH (Wahhab, 1948). These tests were, however, negative with the precipitate. On further storage for a month at 37° or 55° C., neither the absorption maximum in the ultraviolet region nor precipitation with 2,4-DNPH was observed in the supernatant.

The red colored solutions, taken up in 70% alcohol or mixed with Whatman cellulose powder and stored at 37° or 55° C., became light brown in one month. Ether extractives of the brown color taken up in water had an absorption maximum at 282 mμ and gave an immediate precipitate with 2,4-DNPH, indicating the presence of carbonyl compounds. Tests for furfural and hydroxymethylfurfural were negative.

Effect of Drying Conditions of Cabbage on AA, Amino Acid, and Color. Data obtained in these studies are presented in Table III. Blanching, generally practiced in the dehydration of vegetables, resulted in significant oxidation of AA, besides losses due to leaching, and enhanced the extent of brown color formed in cabbage dried in a cabinet dryer. Sulfiting did not prevent the oxidation of AA or subsequent reaction with amino acids during drying, although it inhibited the discoloration in the dried product. As drying proceeded, the extent of oxidation of AA increased. Half-way in the process of drying, the losses in

Table II. Color Formation in Aqueous and Alcoholic Media

Glycine, Mg./10 Ml.	AA/DHA/DKG, Mg./10 Ml.	Medium	Absorbance, Color at 520 Mμ
	DHA		
1.5	6.96	80% Alcohol	4.2
3.0	6.96	80% Alcohol	7.2
4.5	6.96	80% Alcohol	9.6
	DKG		
1.5	7.68	80% Alcohol	1.1
3.0	7.68	80% Alcohol	1.7
4.5	7.68	80% Alcohol	2.4
	AA		
250	50	Water	1.8
250	100	Water	5.4
250	150	Water	9.6

Table III. Changes in Amino Acids, AA, and Color during Drying of Cabbage

Mode of Drying	Treatment	Drying Period, Hours	Moisture Content, %	Ascorbic Acid (M.F.B.),[a] Mg./100 G.	DHA + DKG (M.F.B.), Mg./100 G.	Free Amino Acids Expressed as Glycine (M.F.B.), G./100 G.	Absorbance, Color at 440 Mμ
Control	Fresh	0	92.75	852.3	120.3	5.27	...
	Blanched	0	92.02	567.0	302.6	4.57	...
	Blanched and sulfited	0	93.39	373.6	367.0	4.57	...
Freeze-dried	Fresh	10[b]	39.92	376.0	338.1	2.82	...
	Fresh	18	2.71	359.0	228.7	2.56	0.08
	Blanched	10[b]	74.14	469.1	299.7	1.96	...
	Blanched	18	3.06	389.4	64.2	1.79	0.03
	Blanched and sulfited	10[b]	80.61	345.3	97.8	...	...
	Blanched and sulfited	18	2.14	312.4	42.0	1.57	...
Hot-air dried	Fresh	4.5[b]	68.80	553.1	570.5	3.09	...
	Fresh	8	12.24	340.5	51.5	2.04	0.11
	Blanched	4.5[b]	52.45	250.8	356.9	1.75	...
	Blanched	8	5.90	131.7	5.7	1.33	0.24
	Blanched and sulfited	4.5[b]	66.36	335.3	183.1	1.52	...
	Blanched and sulfited	8	9.43	223.5	36.2	1.34	0.04

[a] M.F.B. = moisture-free basis.
[b] Estimations carried out in the intermediate stage of drying.

AA were reflected in a nearly corresponding increase of its oxidation products—DHA and DKG. In the final product, further loss of AA was noted but no corresponding increase of DHA and DKG. On the contrary, a considerable loss of DHA and DKG occurred, which was very high in hot air drying as compared with freeze drying. A considerable loss in the amino acid content was also noted which, however, was more than the losses of AA and its oxidation products. This may be due to the reaction of amino acids with sugars as observed by Frumkin and Petrash (1962) in dried cabbage on storage. Low temperature and minimal oxidation of AA in freeze drying resulted in a light red color, while in hot air drying, high temperature and considerable oxidation caused a brown color. Even then, cabbage dried in the cabinet dryer without blanching had only a light reddish tinge, and the extent of browning was less. Ether extractives of freeze-dried or hot air-dried samples, either fresh or stored (200 days at 24–26° C.), had no absorption maximum in the ultraviolet region, and the tests for furfural and hydroxymethylfurfural were negative.

DISCUSSION

Investigations on the identification of the reactants responsible for the development of the red color in freeze-dried or in stored alcoholic extracts of fresh cabbage and studies in model systems show that the free amino acids present in cabbage react with the oxidized products of AA (DHA and DKG) to cause discoloration. Formation of aldehyde and carbon dioxide in the amino acid–AA reaction mixture indicates that the mechanism involved is Strecker degradation. A positive test for free $-NH_2$ and $>C=O$ groups in the red colored solutions indicates that it is the rearranged product of Schiff bases, the formation of which is evident from the mechanism reported by Schönberg and Moubasher (1952).

Amino acid–AA interaction takes place under relatively low moisture conditions. In an aqueous medium, the reaction does not take place in low concentrations of the reactants (Table I), and even when it takes place in high concentrations, the product of reaction is unstable, as is evident from the breakdown of red color stored in aqueous medium at 37° C. The alcoholic medium in model systems provides the low moisture conditions required.

The aldehydes formed by the Strecker degradation are a source of browning (Hodge, 1953). Liberation of a carbonyl group, when the red color changes to brown during storage in 70% alcohol or on cellulose, indicates that browning also involves the breakdown of Schiff bases to carbonyl compounds.

The AA-induced browning in citrus juices and concentrates, which initially involves its decomposition to furfural and subsequent polymerization or reaction with amino compounds, is dependent on pH, and within the pH range of 2.0 to 3.5 the extent of browning is inversely proportional to pH (Braverman, 1963). Although, in aqueous systems, this may be the mechanism at a low pH, as furfural is the main product of decomposition (Huelin, 1953); in dried cabbage, similarity in the absorption maximum of the color to that of the model systems (Figure 4), losses in DHA, DKG, and amino acids, a positive test for $-NH_2$ and $>C=O$ groups in the red color, and the absence of furfural either initially or during storage indicate that the mechanism of discoloration is similar to that observed in the model systems and involves Strecker degradation. Formation of DHA and DKG from AA during drying, low moisture, and high concentration of the reactants (amino acids, DHA, and DKG) in the last stages of drying favor their interaction. The pH of cabbage (5.2) is optimum for this. Since the color formation in the amino acid–DHA and DKG system does not occur below pH 3.5, and as the pH of most of the vegetables is in the optimum range (5.3 to 6.5) required for their interaction and is unfavorable for furfural formation (at pH 4.0 and above) (Huelin, 1953), this mode of discoloration should be considered in other dehydrated vegetables.

ACKNOWLEDGMENT

The authors are indebted to M. V. Patwardhan and S. P. Manjrekar for their suggestions, to other members of the laboratory for counsel and assistance, and to H. A. B. Parpia for his keen interest and discussions during the course of this work.

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Shamanna Ranganna
Lakshminarayana Setty

Central Food Technological
Research Institute
Mysore-2, India

Received for review August 28, 1967. Accepted January 30, 1968.