Chlorophyll pigments and their relation to food colour

Author:
Buckle, Kenneth Alan

Publication Date:
1968

DOI:
https://doi.org/10.26190/unsworks/5660

"CHLOROPHYLL PIGMENTS AND THEIR RELATION TO FOOD COLOUR"

Presented as a Thesis for the degree of

Doctor of Philosophy

of

The University of New South Wales

by

Kenneth Alan Buckle B.Sc.(Hons)

Submitted

SYDNEY, November 1968

D E C L A R A T I O N

The candidate, Kenneth Alan Buckle, hereby declares that none of the work presented in this thesis has been submitted to any other University or Institution for a higher degree

A C K N O W L E D G M E N T S

The author wishes to thank Dr. R.A. Edwards, Senior Lecturer, Department of Food Technology, for his keen interest in this investigation and his valuable guidance in the preparation of the manuscript.

A grateful acknowledgment is made to Mr. P.C. Thompson, Gordon Edgell Pty. Ltd., for obtaining raw materials used in these investigations.

The author would also like to thank Mr. W.R. Day, Professional Officer, Department of Food Technology, for his helpful technical advice; Professor M. Chaiken, Head, School of Textile Technology, for the use of spectrophotometric facilities in his laboratories; and Mr. C. Gray, C.S.I.R.O. Division of Computing Research, for statistical analyses.

Grateful acknowledgment is made to the Directors of General Motors-Holden Pty. Ltd. for the award of a G.M.-H. Postgraduate Research Fellowship, and to the University of New South Wales for the award of a University Postgraduate Research Scholarship.

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S U M M A R Y

A summary of the nature of the chlorophyll pigments is followed by a review of their chemical reactions and methods for their isolation and quantitative determination. A brief report on psychophysical aspects of colour and systems of colour notation leads to a review of objective colour measurements of chlorophyll-containing foods.

Experiments are described which show significant correlations between percent chlorophyll conversion to pheophytin and objective colour indices derived from tristimulus measurements on stored pea puree processed by conventional and H.T.S.T. methods, with or without pH elevation. The effects of storage time and temperature, product pH and process temperature on pigment composition, colour and pH are discussed, and process combinations required for adequate colour retention during storage are defined.

Unusual pigment results obtained during analyses on processed and stored elevated pH pea puree are shown, by detailed chromatographic examination, to be caused by the presence of three pigments not previously reported in processed foods. The three pigments, designated pigments X,Y and Z, appear to be derived from pheophytins *a* and *b* and chlorophyll *b* respectively, and are characterised, after isolation and purification, in terms of UV-visible and infrared spectra, and chemical tests. Possible mechanisms of formation of the three pigments are discussed in relation to known chemical reactions of chlorophyll pigments.

Pigments obtained after incubation of pheophytins *a* and *b* and chlorophyll *b* in model systems containing sodium bicarbonate or magnesium

carbonate are shown to be different from pigments X,Y and Z isolated from stored pea puree, and the significance of the reactions involved are discussed.

Several relationships are shown to exist between chlorophyll pigments, colour, pH and lipid oxidation in stored frozen peas and beans. Chlorophyll conversion and objective colour indices are shown to correlate closely during the frozen storage of both materials. Changes in pigments, colour, pH and lipid oxidation in unblanched peas are shown to be caused by the activity of lipoxygenase and chlorophyll-bleaching enzyme systems.

Model system studies on chlorophyll bleaching with soyabean and fresh pea enzyme extracts implicate a lipohydroperoxidase-type factor as responsible for total pigment losses in unblanched frozen peas. Possible mechanisms of pigment and colour changes in frozen vegetables are discussed.

The experimental methods and results are tabulated and a bibliography is given.

1. INTRODUCTION

The preservation of food materials by various processing techniques invariably results in changes in quality characteristics such as colour, flavour or texture. One of the most important quality attributes is colour, since this is a very evident characteristic of food. The acceptability of a food product by a consumer is dependent on a favourable visual impression based on preconceived ideas of "acceptable" colours for different food products.

Colour can be an obvious indicator of a lack of a desired quality characteristic in a food, particularly with foods that undergo a colour change during maturation or ripening. This is so for fruits such as peaches and tomatoes, where yellow or bright red colours respectively together with the absence of green colours, are taken as evidence of quality by both processors and consumers. In some countries, e.g. South Africa and the United States, standards of quality for various attributes, including colour, have been established for the grading of agricultural and food products. Colour can also indicate the development of undesirable characteristics in a food, e.g. the enzymic browning of cut apple tissue, and in such cases the consumer may associate these changes with nutritional defects, spoilage, or a general lowering of acceptability. Thus particular colours are associated with acceptable foods, and foods of a colour different to that expected may be rejected as being undesirable or of unacceptable quality.

Food products containing the green chlorophyll pigments are particularly susceptible to such types of discrimination since such changes in

quality are usually manifested by undesirable colour changes. Since the introduction of heat processing as a method of food preservation, considerable attention has been focussed on attempts to prevent or diminish adverse colour changes both during processing and post-process storage. Early attempts to prevent colour changes by a control of product pH were not successful, and for many years the problem remained unsolved. Recent developments in quality retention during processing and storage have centred on high temperature-short time (H.T.S.T.) processing, in combination with pH elevation, as a means of producing high quality products with a minimum of colour deterioration. Although such processes have developed products superior to conventional products in terms of colour and flavour, evidence is lacking concerning the long term storage behaviour of pH-elevated, H.T.S.T.-processed materials containing high concentrations of chlorophyll pigments. The aim of Part I of the present investigation was to examine the influence of storage time and temperature on the chlorophyll pigment composition, colour and pH of H.T.S.T. processed pea puree.

The upsurge in popularity of frozen foods in recent years has resulted in many investigations on chlorophyll-containing vegetable products and the factors contributing to colour changes during frozen storage. Recent reports have implicated fat oxidation mechanisms as being partly responsible for colour and flavour changes in stored frozen peas and beans. Part II of this investigation was designed to examine the mechanisms involved in colour deterioration during the frozen storage of such materials. Attempts were made to correlate lipid

oxidation and enzymic processes with observed changes in colour and chlorophyll pigments as an aid to understanding more fully some of the changes which take place during frozen storage.

2. LITERATURE REVIEW

2.1 CHLOROPHYLL PIGMENTS

Chlorophyll is the general name for the green pigments in plants, some and perhaps all of which participate in the process of photosynthesis. The importance of photosynthesis has stimulated much research and has resulted in the accumulation of a vast amount of knowledge concerning the process itself and the pigments responsible for the primary act, the absorption of light energy. Chlorophyll-type pigments have been found in higher plants; red, green and brown algae; diatoms; dinoflagellates; and photosynthetic green, brown and purple bacteria. Various aspects of chlorophyll pigments have been treated in several monographs and reviews, including those of Willstatter and Stoll (1913), Corwin (1943), Steel (1943), Rabinowitch (1945,1951,1956), Aronoff (1950a,b,1953,1960,1966), Smith and Benitez (1955), French (1960), Livingston (1960), Braverman (1963), Hill (1963), Smith and French (1963), Goodwin (1965) and Vernon and Seely (1966).

2.11 Classification of Chlorophylls

For some time there was thought to be but a single pigment in photosynthetically active plants, namely the green of leaves or chlorophyll. However, early work showed that green chlorophyll was in fact a mixture of yellow pigments and two different green pigments, the latter referred to as blue-green and yellow chlorophylls. Their separation by column chromatography was achieved by Tswett (1906), who called them chlorophylls α and β. Since this early work, a large number of chlorophyll pigments have been extracted from plants, algae and bacteria. They include

chlorophylls a, b, c, d, e, bacteriochlorophylls a and b, and chlorobium chlorophylls '650' and '660'. Their distribution among photosynthetic organisms has been reviewed by Strain (1958), Holt (1965) and Allen (1966) (Table 2.1).

Table 2.1 Distribution of Chlorophylls Among Photosynthetic Organisms (from Holt (1965)).

	Pigment				
	Chlorophyll				
Organism	a	b	c	d	e
Higher plants, ferns and mosses	+	+	-	-	-
Algae					
Chlorophyta	+	+	-	-	-
Chrysophyta					
Xanthophyceae	+	-	-	-	+
Chrysophyceae	+	-	+	-	-
Bacillariophyceae	+	-	+	-	-
Euglenophyta	+	+	-	-	-
Pyrrophyta					
Cryptophyceae	+	-	+	-	-
Dinophyceae	+	-	+	-	-
Phaeophyta	+	-	+	-	-
Rhodophyta	+	-	-	+	-
Cyanophyta	+	-	-	-	-

	Bacteriochlorophyll			Chlorobium Chlorophylls		
	a	b		'650'		'660'
Bacteria						
Thio- and Athiorhodaceae	+	or	+	-		-
Chlorobacteriaceae	+		-	+	or	+

2.111 Chlorophylls in Higher Plants and Green Algae

Chlorophylls may be regarded as magnesium complexes of compounds derived from phorbin (Figure 2.1) which is the dihydro derivative of porphin containing the isocylic or cyclopentanone ring V. The chlorophylls found in higher plants and green algae are chlorophylls a and b. The structure of chlorophyll a is given in Figure 2.2 (Fleming 1967), and for chlorophyll b, -CHO replaces $-CH_3$ at C-3. Chlorophyll a ($C_{55}H_{72}O_5N_4Mg$) is thus a magnesium chelate of 1,3,5,8-tetramethyl-4-ethyl-2-vinyl-9-keto-10-carbomethoxy phorbin phytyl-7-propionate. Chlorophyll b ($C_{55}H_{70}O_6N_4Mg$) is a magnesium chelate of 1,5,8-trimethyl-3-formyl-4-ethyl-2-vinyl-9-keto-10-carbomethoxy phorbin phytyl-7-propionate.

The structure of chlorophyll a (and hence chlorophyll b) is now known in considerable detail. The relative configuration of the methyl and propionic ester groups on ring IV was shown to be trans by Ficken et al.(1956). The absolute configuration of the long-chain alcohol phytol ($C_{20}H_{39}OH$) esterified to the propionic acid group attached to C-7 has also been shown to be trans; i.e. phytol is 3,D-7,D-11,15-tetramethyl hexadec-trans-2-en-1-ol (Figure 2.2) (Burrell et al.1959,1966; Crabbe et al.1959). Recently, Wolf et al.(1967) showed that the relative configuration at C-10 is that in which the methoxy-carbonyl group is trans to the propionic ester side chain on C-7. Fleming (1967) has recently reported the absolute configuration of chlorophyll at C-7 and C-8, enabling the complete structure of chlorophylls a and b to be described. Previously two diastereoisomeric structures were possible.

The chlorophylls, in vivo, are found in leaf structures called

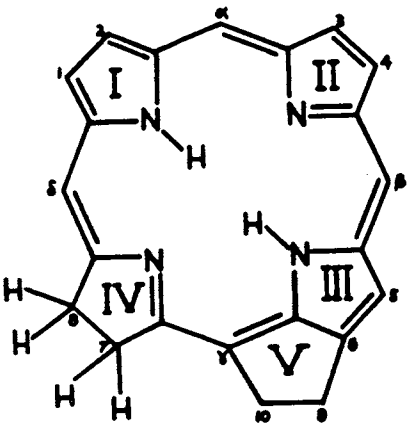


FIGURE 2.1

Phorbin

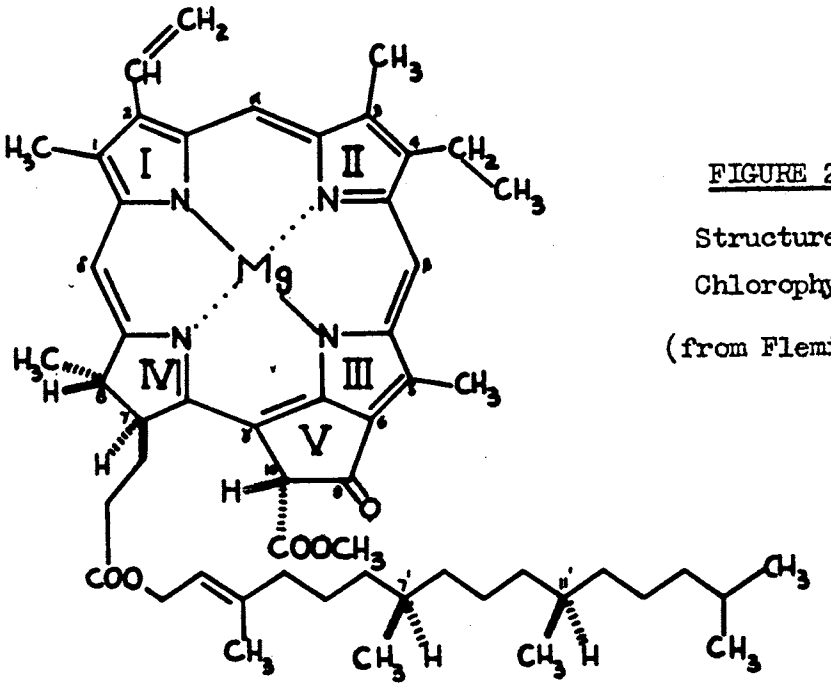


FIGURE 2.2

Structure of
Chlorophyll a
(from Fleming 1967)

chloroplasts associated with protein and lipid material, and give rise to absorption spectra differing considerably from the spectra of pigments after extraction with organic solvents. The various spectra of chlorophylls *in situ* are probably caused by combination of pigments with different proteins, the complexes being destroyed by extraction with organic solvents (French 1958). The various forms of chlorophyll *a* and *b* *in vivo* have been extensively investigated by several workers, and are reviewed by Brown (1963) and Deroche and Costes (1966).

Strain and Manning (1942a) reported the presence of chlorophyll *a*^{1} and *b*^{1}, isomeric forms of chlorophylls *a* and *b* separable on sugar columns. As they both gave magnesium-free derivatives different from those of the parent chlorophylls, they did not differ with respect to the attached magnesium. Later reports have indicated that they may be optical isomers at C-10 of the parent chlorophylls (Pennington et al.1964; Bacon and Holden 1967a).

In higher plants, chlorophylls *a* and *b* are normally found in the ratio of about 2.5 or 3 to 1 (a : b). Many factors influence the ratio of the two pigments, including the species, physiological state of the material, growing conditions, and environment such as light intensity and day length. The chlorophyll *a*/*b* ratio drops slowly during ageing of leaves (Sestak 1966b).

2.112 Other Plant Chlorophylls

Characterisation of chlorophyll-type materials from plant and bacterial sources has shown the presence of a large number of different pigments (Table 2.1). Among higher plants and algae, chlorophyll *a* is

universally found together with chlorophyll b or other minor pigments chlorophylls c, d or e (Strain 1958).

2.1121 Chlorophyll c

Chlorophyll c was found associated with chlorophyll a in brown algae (Phaeophyta) and diatoms (Bacillariophyta) by Strain and Manning (1942b). It is also found in dinoflagellates (Pyrrophyta) and in symbiotic algae and sea anemones (Strain et al.1943), and in other marine algae (Allen 1966). Chlorophyll c is the least known of the major photosynthetic pigments, although organisms containing this pigment carry out most of the photosynthesis in the oceans, playing the same dominant role as the green plants on land. Concentrations of chlorophyll c, in some marine organisms, are as high as that of chlorophyll a (Allen 1966).

Chlorophyll c has been obtained sufficiently free from other pigments for spectral analyses (Haxo and Fork 1959) and has been crystallised after purification by column chromatography (Jeffrey 1962,1963). Recent work has shown that chlorophyll c, isolated from the marine diatom Nitzschia closterium (Strain and Svec 1966), is a mixture of two related phytol-free chlorophyll derivatives, magnesium hexadehydropheoporphyrin a_5 monomethyl ester and magnesium tetradehydropheoportphyrin a_5 monomethyl ester (Dougherty et al.1966).

2.1122 Chlorophyll d

Chlorophyll d is often considered to be characteristic of the red algae (Rhodophyta), although it is by no means universally present in this group. It was isolated from extracts of species of the red alga Gigartina (Manning and Strain 1943), and its preparation has been

described by Smith and Benitez (1955). Comparison of chlorophyll d with a pigment obtained by permanganate oxidation of chlorophyll a showed chlorophyll d to be 2-desvinyl-2-formyl chlorophyll a (Holt and Morley 1959; Holt 1961).

2.1123 Chlorophyll e

Chlorophyll e has been found only twice in members of the Xanthophyta (yellow-green algae), and could not be detected in these species after culturing (Strain 1951). The identity of the pigment has yet to be determined.

2.113 Bacterial Chlorophylls

2.1131 Bacteriochlorophylls a and b

Bacteriochlorophyll a is present in cells of purple and brown photosynthetic bacteria and the purple sulphur bacteria. It also occurs in the green sulphur bacteria together with other chlorophyll pigments. It has been converted into compounds showing similarities in structure to those derived from chlorophyll a (Fischer and Stern 1940). Compared to chlorophyll a, bacteriochlorophyll a has ring II reduced at C-3 and C-4 in a trans configuration (Golden et al.1958), and the vinyl group at C-2 is oxidised to $-COCH_3$. Bacteriochlorophyll b has only recently been discovered in a Rhodopseudomonas species and a related organism (Jensen et al.1964). From spectral changes on reduction, the pigment appears to possess the 2-formyl group (Holt 1966).

2.1132 Chlorobium Chlorophylls

Green photosynthetic bacteria have been shown to contain a large number of different chlorophylls. Stanier and Smith (1960) showed that

different strains of the green bacterium Chlorobium thiosulphatophilum contained two different series of chlorophylls with absorption maxima in the red at 650 and 660nm in diethyl ether. Various aspects of the structure of Chlorobium chlorophylls '650' and '660' have been investigated by Holt and coworkers (Holt 1965,1966), who found up to 6 fractions in each type. Rapoport and Hamlow (1961) have shown that both series of pigments do not contain phytol, but a 15-carbon alcohol farnesol.

2.12 Function of Chlorophylls

The importance of the process of photosynthesis in the biological world has been recognised for many years. The process of photosynthesis involves the production of organic compounds from the raw materials carbon dioxide, water and energy. From a chemical standpoint, the process could be written thus:

$$\text{Sunlight (energy)} + \text{(Pigments)} + XCO_2 + XH_2O \longrightarrow \text{(Pigments)} + (CH_2O)_X + XO_2$$

The net result is a transformation of electromagnetic energy of absorbed light to chemical potential energy of the synthesised carbon compounds. The absorption of energy is carried out by the molecules of chlorophyll in association with other chloroplast pigments (e.g. carotenoids). The detailed photochemical and biochemical mechanisms involved in light adsorption and energy conversion have not, as yet, been entirely solved. Extensive reviews of such aspects of chlorophyll function are given by San Pietro and Black (1965), Vernon and Avron (1965), Whittingham (1965) and Clayton (1966).

2.121 Chloroplast Structure

Although the chlorophyll pigments have long been known to be located

in the sub-cellular chloroplast structures, at least within leaves of higher plants, there is still doubt as to their precise chemical environment. The importance of this question lies in the premise that only when the structural relation of chlorophylls to other chloroplast molecules is known will it become possible to describe fully the mechanism by which chlorophyll takes part in the conversion of light energy to chemical energy. Much of the macromolecular organisation of chloroplasts has been revealed in recent years by studies with the electron microscope and other techniques. Various aspects of chloroplast structure have been reviewed in the literature by Wassink (1963), Wolken (1963) and Park (1966).

2.122 Chlorophyll - Protein Complexes

It has long been recognised that the state of chlorophyll *in vivo* is strikingly different from that of extracted chlorophyll in solution. The properties ascribed to chlorophyll in plants and in fresh aqueous extracts of plants are not those of the chemically defined substance. Several lines of indirect evidence point to an association of chlorophyll with protein and lipid (Kupke and French 1960). Early attempts to extract native chlorophyll-protein complexes from plant materials have been reviewed by Goedheer (1966). These studies have produced photochemically inactive complexes (Smith 1942) as well as active preparations (Kahn and Chang 1965; Kahn and Bannister 1965; Anderson and Boardman 1966; Boardman and Highkin 1966). The properties of many of these complexes have been reviewed by Goodwin (1966).

2.13 Synthesis of Chlorophylls

2.131 Synthesis in vitro

One of the exceptional accomplishments in organic chemistry in recent years has been the total synthesis of chlorophyll a by two independent groups of workers. This was achieved by Woodward and coworkers at Harvard (Woodward 1960,1961; Woodward et al.1960) and by Strell and coworkers at Munich (Strell et al.1960). This work brought to completion the immense body of information obtained by Fischer's school in their work on pyrroles (Fischer and Orth 1937; Fischer and Stern 1940). The work of Fischer and Woodward on the synthesis of chlorophyll a has recently been reviewed by Lwowski (1966). Phytol, the long chain alcohol attached to the propionic acid group at C-7 in chlorophylls a and b, has recently been completely synthesised by Sato et al.(1967).

2.132 Biosynthesis

Following on the work of Willstatter, Fischer and others on the basic chemistry of heme and chlorophyll, it soon became apparent that a common pathway of biosynthesis existed for these pigments. Work during the last two decades has verified this assumption. The main steps of chlorophyll biosynthesis have now been identified, although complete enzymic studies of some isolated reactions are lacking. Excellent reviews covering the biosynthetic pathway of chlorophylls have been published by Bogorad (1965,1966), Granick (1965) and Marks (1966).

2.2 THE CHEMISTRY OF CHLOROPHYLL PIGMENTS

The chemistry of chlorophyll pigments has been extensively investigated during the past half century for many reasons. Early work con-

sidered mainly their close structural relationship to the biologically important heme compounds, but for some time, particularly in recent years, this has been overshadowed by research into their functional role in photosynthesis. However, the influence of colour in determining consumer acceptability of processed foods has also focussed some attention on the reactions of chlorophyll pigments, particularly on their stability and degradation during processing and storage. Various aspects of chlorophyll chemistry have recently been reviewed by Holt (1965), Seely (1966) and Holden (1967b). This work reviews only those aspects of the chemistry of chlorophylls a and b that are of importance in relation to the colour of chlorophyll-containing materials as affected by processing techniques, and insofar as such information is pertinent to the analysis and identification of chlorophyll derivatives.

Terms and nomenclature associated with chlorophylls and their derivatives are presented below (Holt 1965; Seely 1966).

1. Porphyrin - In the broad sense, any macrocyclic tetrapyrrole pigment in which the pyrrole rings are joined by methine bridges and the system of double bonds forms a closed, conjugated loop. In the narrow sense, porphyrin designates substituted porphins, as distinct from chlorins.
2. Porphin - Term given by Fischer and Halbig (1926) for the unsubstituted, fully unsaturated porphyrin.
3. Pyrrole - The four cyclic components of the porphyrin nucleus.

4. Chlorin - In the broad sense, any 7,8-dihydroporphin. It has been conventional to distinguish 4 subclasses: (i) Rhodins - chlorins of the b series. (ii) Phorbins (or phorbides) - having an isocyclic or cyclopentanone ring V. (iii) Purpurins - having an electron attracting group, such as carbonyl, at the δ position. (iv) Chlorins - in the narrow sense, are 7,8-dihydroporphins with a heterocyclic closure between the C-6 and δ positions (e.g. a lactone).

5. Phyllins - Magnesium complexes of chlorins.

6. Pheophytins - Magnesium-free chlorophylls.

7. Chlorophyllides - Phytol-free chlorophylls.

8. Pheophorbides - Magnesium-free chlorophyllides (or phytol-free pheophytins).

9. Pyro compounds - Derivatives in which the C-10 carbomethoxy group has been replaced by a hydrogen atom.

10. Meso compounds - Derivatives in which the C-2 vinyl group has been reduced to an ethyl group.

The relationship between the chlorophylls, pheophytins, chlorophyllides and pheophorbides of the a and b series is given in Table 2.2.

Table 2.2 Relation Between Chlorophyll Derivatives

Pigment	Symbol	Magnesium	Phytol	C-3 Methyl	C-3 Formyl
Chlorophyll a	C_a	+	+	+	-
Chlorophyll b	C_b	+	+	-	+
Chlorophyllide a	Cd_a	+	-	+	-
Chlorophyllide b	Cd_b	+	-	-	+
Pheophytin a	Py_a	-	+	+	-
Pheophytin b	Py_b	-	+	-	+
Pheophorbide a	Po_a	-	-	+	-
Pheophorbide b	Po_b	-	-	-	+

2.21 Reactions of Chlorophylls

2.211 Reaction with Acid

The chlorophyll pigments are relatively stable compounds *in vivo*. When the physicochemical environment in the cell is changed by cell death or extraction with organic solvents, the pigments become less stable and undergo degradation reactions by various mechanisms. Green chlorophyll solutions rapidly turn olive green and brown after treatment with dilute acids. This reaction is known to involve the replacement, with hydrogen, of the centrally-bonded magnesium in the porphyrin ring, forming the corresponding pheophytin (Willstatter and Hocheder 1907).

The kinetics of the conversion of chlorophylls a and b to the respective pheophytins was first studied by Willstatter and Stoll (1918), who found that colloidal solutions of chlorophyll a in water were decomposed 80% in 24 hours by carbon dioxide, while chlorophyll b was decomposed only 25% in 48 hours. These investigations were continued by

Joslyn and Mackinney (1938), who used 90% aqueous acetone solutions containing a mixture of chlorophylls a and b, and measured the appearance of a prominent band at 535nm for pheophytin a and at 528nm for pheophytin b. Using acid concentrations of 10^{-2} to $2x10^{-4}$N, they concluded that the rate of conversion of chlorophyll to pheophytin in 90% acetone was of first order with respect to acid concentration and possibly second order with respect to chlorophyll concentration. The first order dependence of acid concentration for the conversion of chlorophylls a and b to the respective pheophytins was confirmed by Mackinney and Joslyn (1940) using oxalic acid and pure chlorophyll solutions. They concluded that the hydrogens replacing magnesium possibly enter at different rates, the entrance of the first H^+ ion being the rate-determining step. Chlorophyll a was found to react 7 to 9 times faster than chlorophyll b.

The temperature coefficient of the rate of pheophytin formation was studied at temperatures of $0^{\circ}C$ to $51^{\circ}C$ by Mackinney and Joslyn (1941). Using oxalic acid, they found activation energies of 7,500 and 9,000 calories per mole for chlorophylls a and b respectively at the lower temperatures, and considered the difference to be due to steric hindrance in the case of chlorophyll b. This seemed possible since Weast and Mackinney (1940) had shown that the enzymic hydrolysis of phytol from chlorophyll b also occurred less readily than from chlorophyll a.

Schanderl et al.(1962) studied the kinetics of the reaction of chlorophylls a and b and the ethyl, methyl and free chlorophyllides with H^+, each in a system (80% acetone, 20% dilute hydrochloric acid) which was 10^{-4}N in hydrochloric acid, at temperatures of $25^{\circ}C$, $35^{\circ}C$, $45^{\circ}C$ and

55°C. The pigments used were highly purified and freshly prepared. These workers found that chlorophyll b reacted only 5.5 times slower than chlorophyll a, as compared to the 7 to 9 times slower rate reported by Mackinney and Joslyn (1940). They also concluded that changing the length of the alcohol esterified with the propionic acid in the C-7 position only slightly affected the ease with which the central magnesium was replaced by hydrogen, indicating that steric hindrance plays only a minor role in the replacement reaction.

The kinetics of the acid catalysed pheophytinisation of chlorophylls a and b have recently been re-examined by Cho (1966) in 80:20 v/v acetone: water solvent - HCl and in 80:20 v/v acetone:water solvent-acid buffer systems. He showed that contrary to previous reports, this reaction is second-order with respect to hydrogen ion concentration and first order with respect to chlorophyll concentration. A new mechanism for the reaction was proposed, in which a rapid equilibrium is established between chlorophyll and the two equivalent hydrogen-ions forming an intermediate in which the hydrogen ions are attached to the nitrogen atoms of the pyrroles. This fast step is followed by a slow rate-determining step in which pheophytin is produced by the loss of magnesium. The slower conversion of chlorophyll b to pheophytin b, compared to the conversion of chlorophyll a to pheophytin a, is thus explained by a smaller value of the equilibrium constant for the fast reaction step of the mechanism. The resonance contributions of chlorophyll b give a more positive charge to the two nitrogen atoms of the pyrroles, and consequently are responsible for the smaller equilibrium constant of the first reaction.

Esters of the C-7 propionic acid group in chlorophyll derivatives are hydrolysed by cold concentrated hydrochloric acid more rapidly than esters of other acid groups, thus pheophorbides a and b can be prepared from the corresponding chlorophylls or pheophytins. The two nitrogens of metal-free porphyrins which do not bear hydrogens are more strongly basic than the other two. The ability of chlorophyll degradation products to form water-soluble mono- and di-protonated species enabled Willstatter to separate them by differential extraction and to characterise them by their hydrochloric acid number (Willstatter and Mieg 1906).

The hydrochloric acid number was defined as the percent composition of the acid which extracted two-thirds of the pigments from an equal volume of ether, and was particularly useful for determining the presence of phytol in chlorophyll-type pigments. Pheophorbides a and b were extracted into 22% HCl whereas the corresponding pheophytins remained in the ether phase (Willstatter and Stoll 1913). Hydrochloric acid numbers of various chlorophyll derivatives are given in Table 2.3 (Holt 1965; Seely 1966). Hydrochloric acid numbers increase with esterification and with the size of the esterifying alcohol, and are larger in the b series than in the a series.

Table 2.3 Hydrochloric Acid Numbers of Chlorophyll Derivatives

Chlorophyll Derivative	HCl Number
Pheophytin b	35
Pheophytin a	29
Methyl pheophorbide b	21
Pheophorbide b	19.5
Methyl pheophorbide a	16
Pheophorbide a	15
Rhodin g_7 trimethyl ester*	12-13
Rhodin g_7*	9
Chlorin e_6 trimethyl ester*	8
Chlorin e_6*	3

from Holt (1965), Seely (1966).

* Subscripts denote the number of oxygen atoms in the molecules.

2.212 Reaction with Alkali

Willstatter and coworkers showed that irreversible changes occurred when chlorophylls were allowed to stand in alcohol solution in contact with air (Willstatter and Stoll 1911). The process was called allomerisation because the properties of the chlorophyll were altered while the appearance and chemical composition seemed to undergo no change. Subsequently, allomerisation was shown to require one mole of oxygen per mole of chlorophyll (Conant et al.1931), and to be due to a slow process of oxidation involving the removal of -H at C-10 and its replacement by -OH or an alkoxy group when alcohols are used as solvents. The reactivity of the C-10 hydrogen is increased in alkaline solutions, indicating the dependence on enol formation at C-9.

Allomerised chlorophylls do not give a positive phase test, first described by Molisch (1896). This test consists of layering 30% (w/v) KOH in methanol under an equal volume of an ether solution of the pigment. In a positive test, a coloured ring is formed at the interface of the two phases, yellow in the a series and red in the b series. For a natural mixture of the two pigments, a yellow-brown ring is formed. After a few moments, the green colour begins to reappear and soon all the pigments are extracted into the aqueous layer, leaving the ether colourless if no carotenoids are present. The phase test is positive with chlorophylls, pheophytins, chlorophyllides and pheophorbides, but is negative with pyro compounds which do not contain the carbomethoxy group attached to C-10. The phase test is positive only with compounds that contain the cyclopentanone ring V, a C-10 hydrogen, and a C-10 carbomethoxy group.

The mechanism of the phase test is believed to involve an enolate ion or an ionised diradical resulting from ionisation of the hydrogen at C-10 (Weller 1954; Holt 1958). Holt (1958), in an investigation of the chemical and spectral properties of allomerised phyllins, obtained support for the hypothesis that the course of allomerisation ordinarily involves oxidation of traces of the Molisch phase test intermediate, which in turn is generated by traces of alkali in solution. By chromatographic isolation and identification of three components from allomerised methyl chlorophyllide a, he showed that formation of one of the isolated fractions could not be accounted for by the mechanism of chlorophyll a allomerisation initially proposed by Fischer and Pfeiffer (1944).

Alkaline hydrolysis of chlorophyll produces magnesium chlorin e_6,

also known as phyllin e_6 and chlorophyllin (Willstatter and Mieg 1906). Hot rapid saponification of pheophytins a and b in strong alkali removes phytol to yield chlorin e_6 and rhodin g_7 respectively. Boiling dilute methanolic KOH or diazomethane-methanol produces chlorin e_6 trimethyl ester or the corresponding rhodin compound. The quantitative cleavage by diazomethane-methanol is considered to be the most reliable test for determining the absence of allomerised pigments.

2.213 Reaction with Enzymes

The chlorophyll pigments may undergo degradative reactions under the influence of enzyme systems both *in vivo* and *in vitro*. Three of those involved are: (i) chlorophyllase; (ii) glycollate oxidase, and (iii) lipoxygenase.

2.2131 Chlorophyllase

Chlorophyllase, or chlorophyll-chlorophyllido-hydrolase (E.C. 3.1.1.14), was discovered over 50 years ago by Willstatter and Stoll (1910), although the products of its action were first observed but not recognised by Borodin (1882). Chlorophyllase *in vitro* catalyses the removal of phytol from chlorophylls a and b and pheophytins a and b, the presence or absence of magnesium apparently not being connected with specificity (Holden 1961,1963). Chlorophyll b is hydrolysed more slowly than chlorophyll a (Mayer 1930; Sironval 1954). Fischer and Lambrecht (1938) tested chlorophyllase activity on a range of chlorophyll derivatives and concluded that the carbomethoxy group at C-10 and the hydrogen atoms at C-7 and C-8 were essential for attack by the enzyme. This would account for the lack of activity with protochlorophyll (Sud'ina 1961). Shimizu

and Tamaki (1963) recently have been able to phytylate chlorophyllide and pheophorbide *in vitro* using a purified preparation of tobacco leaf chlorophyllase.

In vivo, it now appears that chlorophyllase is responsible for the final stage of chlorophyll biosynthesis, i.e. the attachment of the phytyl side chain to chlorophyllide (Sud'ina 1963). Chlorophyllase has been found to be widely distributed in flowering plants (Mayer 1930), where the activity is concentrated in the chloroplast fraction of leaves, probably in a chlorophyll-lipoprotein complex (Ardao and Vennesland 1960).

Three general methods have been described for the measurement of chlorophyllase activity. The first involves extraction of both chlorophyll and chlorophyllide from an acetone solution into ether and subsequent extraction of the chlorophyllide by alkali (Peterson and Mackinney 1938); the second is based on the insolubility of chlorophyllides in light petroleum (Holden 1963); and the third involves chromatographic separation of the products of hydrolysis (Gage and Aronoff 1956).

Various aspects of the action and preparation of chlorophyllases have been reviewed by Holden (1965b) and Grob and Seiler (1967).

2.2132 Glycollate Oxidase

This enzyme (glycollate: O_2 oxidoreductase, E.C. 1.1.3.1) catalyses the oxidation of glycollate to glyoxylate and is activated by light in intact etiolated plants. Concurrent with the oxidation of glycollic acid *in vitro* there is a loss of colour of chlorophyll solutions and an uptake of oxygen in addition to the oxygen rapidly utilised in the

oxidation reaction (Kolesnikov 1948,1949; Tolbert and Burris 1950). The chlorophyll is decolourised through an oxidative reaction in which it is claimed that an organic peroxide is produced.

2.2133 Lipoxygenase

The enzyme lipoxygenase or lipoxidase (E.C. 1.13.1.13), found in legumes, some cereal grains and oil seeds, catalyses the peroxidation of unsaturated fatty acids that contain the cis-cis 1,4-pentadiene system, such as linoleic, linolenic and arachidonic acids, esters and glycerides, but not those of oleic acid (Dillard et al.1960, 1961). Some reports have suggested the presence of 2 or more enzymes specific for fatty acids and glycerides (Koch et al.1958; Dillard et al.1961) and recently several isoenzymes have been separated from wheat and soya extracts on polyacrilamide gels (Guss et al.1968). The principal product of lipoxygenase catalysis is an optically active cis-trans conjugated monomeric hydroperoxide (Privett et al.1955), which is different from products obtained from autoxidation of unsaturated acids (Khan 1965).

Lipoxygenase has been studied in great detail because of its theoretical and practical importance. The enzyme is of interest in food processing and preservation because of its role in bleaching carotene in dough, and in the deterioration of colour and flavour of unblanched frozen vegetables. Lipoxygenase was initially discovered via its oxidative action on the secondary substrate β-carotene, and for some time was erroneously called carotene oxidase before the primary action of the enzyme was known. This coupled oxidation has now been extensively investigated (Blain et al.1953; Tookey et al.1958). Various aspects of

the chemistry, isolation, distribution and importance of lipoxygenase have been discussed in the reviews of Tappel (1961,1962a,b) and Reed (1966).

The influence of lipoxygenase on colour and flavour deterioration in unblanched frozen vegetables has been known for some time (Wagenknecht et al.1952; Lee 1954; Wagenknecht and Lee 1956,1958). These workers showed that lipoxygenase and lipase remained active for some considerable time in unblanched peas stored at $0^{\circ}F$, resulting in oxidation of lipids and degradation of chlorophyll pigments. Blanched samples showed little, if any, change. The observed lipid oxidation and subsequent degradation of chlorophyll appeared different from both the photodecomposition of organic peroxides (Yu 1966) and the catalytic effect of light-sensitized chlorophyll on the autoxidation of unsaturated fatty acids (Khan 1959). Organic peroxide photodecomposition has been observed in model systems as well as in prepared foods such as sausage products (Hall and Mackintosh 1964).

Recent work has shown that chlorophyll solutions are rapidly bleached in systems containing unsaturated fatty acids and extracts of legumes containing lipoxygenase (Mapson and Moustafa 1955; Walker 1964a; Holden 1965a,c,1967a,b). Walker (1964a,b) examined chlorophyll degradation in frozen French beans and model systems, and reported that loss of total pigment in frozen beans coincided with the onset of fat peroxidation. Model system studies using bean homogenates as a source of lipoxygenase and crude chlorophyll indicated that chlorophylls were degraded to pheophytins both aerobically and anaerobically, but total pigment was destroyed only after aerobic incubation and not at all under anaerobic

conditions. With pure chlorophyll preparations, there was no conversion of chlorophyll to pheophytin under anaerobic conditions, but aerobically, chlorophylls were destroyed with no lag period. Purified soybean lipoxygenase had a similar effect to bean homogenates on both crude and pure pigment preparations. From these results, Walker postulated that during the anaerobic initiation stage of fat peroxidation, the free radical produced reacted with an unidentified intermediate causing the conversion of chlorophyll to pheophytin, but the aerobic propagation step was considerably inhibited by an antioxidant present in the beans. After its exhaustion, chain propagation commenced and both chlorophylls and pheophytins were destroyed by some aerobic intermediate of the reaction chain. Although lipoxygenase was shown to be destroyed in commercial blanching operations, Walker indicated that over-blanching could initiate fat peroxidation reactions.

Some of Walker's results have been disputed by Holden (1965a) who examined chlorophyll bleaching in model systems using a variety of legumes and legume seed extracts. Holden found that pure lipoxygenase and unsaturated fatty acids did not cause chlorophyll bleaching without the presence of a "bleaching factor" present in the legume seed extracts. Addition of pure lipoxygenase and unsaturated fatty acids to legume seed extracts considerably increased the bleaching. The results of several experiments indicated that bleaching was produced by breakdown of hydroperoxides catalysed by the "bleaching factor", which had properties similar to a lipohydroperoxidase discovered initially in soya extracts (Blain and Styles 1959; Blain and Barr 1961; Gini and Koch 1961). The

bleaching of chlorophyll was different from the coupled bleaching of carotenoids in lipoxygenase-unsaturated fatty acid systems (Blain et al. 1953). Thus in Walker's scheme, bleaching occurred by pigment reaction with a free radical intermediate of the lipid oxidation chain, pure lipoxygenase having the same effect as crude bean homogenate on the formation of hydroperoxide, whereas according to Holden, lipoxygenase produced hydroperoxides which were subsequently broken down, by the "bleaching factor", to reactive compounds which destroyed the pigment molecules.

2.214 Metal Complexing

Although magnesium is readily removed from chlorophylls by dilute acid solutions, the reverse reaction is much more difficult and requires treatment of the pheophytin or pheophorbide with a Grignard reagent in alcohol (Fischer and Goebel 1936). Aronoff (1962) has shown that the magnesium of chlorophyll a does not exchange with ^{28}Mg. Chlorophyll derivatives such as pheophytins and pheophorbides react readily in neutral or acid solutions to form very stable metal complexes. Willstatter and Sjoberg (1924) first investigated copper and zinc complexes of chlorophyll derivatives and noted the appearance of highly coloured products. Later work has been reviewed by Seely (1966). Kim (1967) and Jones et al.(1968) have recently reported analytical data and absorption spectra for various chlorophyll chelates.

The occurrence of regreening in commercially prepared pea puree (Schanderl et al.1965) and green beans (Declaire 1966) has been shown to be due to metal complexing of pheophytins and pheophorbides by small amounts of copper and/or zinc ions.

2.22 Degradation of Chlorophylls in Processed Foods

2.221 Heat Processing

2.2211 Conventional Processing

Heat processing techniques have always played a major role in the preservation of foods. One of the disadvantages of using heat as a means of preservation, however, is the modification of pigments which results in changes in the original colour of the food material. Since visual colour is accepted as a major factor in establishing food quality, a considerable research effort has attempted to define the nature of pigment changes that result from processing and, further, to devise new processing techniques by which such changes can be minimised.

The effect of conventional processing methods on chlorophyll degradation in vegetables has been reported by a number of workers. The loss of chlorophylls and the development of dark colours in stored bean puree was shown by Westcott et al.(1955) to be the result of accumulation of pheophytins and pheophorbides, although Siegele (1955) found pheophytins as the only degradation products in canned green beans. De Weese (1962) reported that the heat processing of snap bean puree resulted in greater destruction of chlorophyll b than chlorophyll a. Bowman and Remmenga (1965) compared chlorophyll conversion to pheophytin in canned beans with that in fresh and frozen beans and found much greater conversion in heat processing. Chlorophyll degradation in spinach has been studied extensively by Tan and Francis (1962), Gupte (1963) and Clydesdale (1966). In most cases, results show that conventional heat processing results in severe loss of chlorophylls together with the formation of unattractive colours.

2.2212 H.T.S.T. Processing

In recent years advantage has been taken of the logarithmic order of thermal death of bacteria as opposed to first order kinetics of chemical and biochemical reactions during thermal processing. A number of high-temperature short-time (H.T.S.T.) thermal processes have been developed, which have been claimed to produce food products of the same bacterial status, but higher quality than produced by conventional heat processing. H.T.S.T. sterilisation has been defined by Ball and Olson (1957): "H.T.S.T. is a sterilisation process where foods are sterilised by heat applied for times ranging from a few seconds to a few minutes. In low acid foods, process temperatures exceed 260^{o}F".

H.T.S.T. processing methods have been shown to have a significant effect on both chlorophyll and colour retention in heat processed foods. Livingston (1957) showed that the colour of several baby food purees sterilised at high temperature was better than that of conventionally processed purees. Brody et al.(1960) and Joffe et al.(1961) studied the effect of H.T.S.T. techniques on chemical and biochemical changes in canned vegetables, and showed that significant quality advantages were obtained by these processes. Epstein (1959) studied the stability of the green colour of H.T.S.T. sterilised peas during processing and storage and showed that the retention of green colour was greater with the H.T.S.T. method than with peas processed in a conventional manner. The effect of process time and temperature on colour and chlorophyll retention in peas and purees was studied by Gold and Weckel (1959). These workers showed that there was a quantitative relationship between

the change in colour of the peas measured by objective methods and the degree of chlorophyll degradation. Adams and Yawger (1961) found that peas given an H.T.S.T. process showed better colour retention after 2 weeks storage than those processed conventionally, but at the end of 4 weeks storage colour differences were minimal.

Several workers have reported the effect of H.T.S.T. processing on colour and pigment retention in spinach puree. Tan and Francis (1962) found that for equivalent sterilising values, increasing the process temperature from 240°F to 280°F resulted in much greater retention of both chlorophyll pigments and green colour as measured objectively by colorimeter. Later work from the same laboratory extended these investigations to include combinations of H.T.S.T. processing and pH adjustment (Gupte 1963; Resende 1966) in conjunction with pre-process conversion of chlorophylls to chlorophyllides (Clydesdale 1966). In all cases, H.T.S.T. methods resulted in better retention of pigments and colour than controls processed by conventional methods. Pigment changes in bean packs during H.T.S.T. processes were studied by Luh et al.(1964) and Resende (1966), and better quality retention was also found in the H.T.S.T. samples.

Although H.T.S.T. processing results in adequate heat treatment so far as bacterial destruction is concerned, several reports have shown that colour, flavour and other quality indices may be effected by enzyme regeneration in materials processed by these techniques (McConnel 1956). Work on peas (Guyer and Holmquist 1954; Farkas et al.1956; Adams and Yawger 1961), beans and spinach (Resende 1966) and other materials have indicated that more severe processing than is given by H.T.S.T. methods

may be needed to completely destroy the enzyme peroxidase. Resende (1966) investigated the effect of H.T.S.T. processing and pH adjustment on peroxidase destruction in bean and spinach purees and concluded that bean puree required considerable over-processing by H.T.S.T. standards in order to obtain complete enzyme inactivation, whereas this was not the case with spinach puree. Where blanching is followed by H.T.S.T. processing, enzyme regeneration would not appear to present a problem.

2.2213 pH Adjustment

Many reports have attributed chlorophyll degradation during heat processing to the liberation of plant acids from ruptured cells, and accordingly, attempts have been made to inhibit such changes by neutralising the acid in the tissue. Probably the best known method is the process for canned peas patented by Blair (1940). The Blair process involved an elevation of the pH of the peas to approximately pH8 by immersion in sodium carbonate solution, followed by blanching in calcium hydroxide and processing in a sugar-salt brine which also contained a suspension of magnesium hydroxide. Although initial chlorophyll retention was found to be about 60% after a H.T.S.T. process at 260°F compared with complete pigment conversion at 240°F, any chlorophyll retained was rapidly lost on storage, and the method was not extensively adopted. Malecki (1957) also described a method for the retention of green colour in processed vegetables. This involved the use of calcium hydroxide and calcium glutamate to control the pH during blanching, and the addition of disodium glutamate and a small quantity of sodium hydroxide to maintain a desirable pH of 8.4-8.8 for 24 hours after canning. This process was found to be

successful with peas, but not with spinach, beans nor particularly, green asparagus.

Gold and Weckel (1959) investigated the effect of pH adjustment on chlorophyll retention in processed pea purees and calculated from chlorophyll degradation rate constants, that 74.1% chlorophyll would be degraded to pheophytins by processing at pH6 and 240°F, while at pH8, only 7.4% degradation would occur. At 280°F and pH8, 1% degradation would be produced assuming instantaneous heating and cooling. These results showed that raising the pH of peas had a definite protective effect upon chlorophyll at low processing temperatures, but when high processing temperatures were used, raising the pH had a negligible effect.

The effect of a combination of H.T.S.T. processing and pH adjustment was used by Gupte (1963), Clydesdale (1966) and Resende (1966) to examine colour and pigment retention in spinach puree. Gupte (1963) showed that increasing the pH with magnesium carbonate or processing by H.T.S.T. methods gave greater pigment retention initially but resulted in considerable degradation in storage. A combination of the two methods, however, gave greater pigment retention than either method alone, but again gave a product which lacked storage stability at room temperature.

Clydesdale (1966) extended work on spinach colour, initially reported by Thomas (1928) and Loef and Thung (1965), by employing a process involving initial conversion of chlorophylls to chlorophyllides with the enzyme chlorophyllase followed by the H.T.S.T.-pH elevation combination reported above. He found no substantial gain in stability when chlorophyll was converted to chlorophyllide prior to processing. Although

considerable retention of chlorophyll(ide) was found when pH adjustment and H.T.S.T. processing was used, which agreed with earlier work, pigment was again lost quickly on storage. The decrease in pH during H.T.S.T. processing was found to be substantially less than that in conventional retort processing. However, during storage at room temperature, H.T.S.T. samples decreased in pH at a faster rate than those processed by conventional methods, a result consistent with previous observations that H.T.S.T. processing produces an initial gain in pigment retention which is rapidly lost upon storage.

2.2214 Cooking

The cooking process, as distinct from heat preservation, has also been shown to affect colour and pigment retention. Gilpin et al.(1959) investigated the effect of thirteen different cooking procedures, including boiling, steaming and pressure cooking, on the acceptability of broccoli and reported that colour and pigments deteriorated significantly when cooked for increasing lengths of time by any of these procedures. Eheart and Gott (1965) studied three methods of cooking on the quality characteristics of green beans and broccoli, and showed that less conversion of chlorophyll to pheophytin occurred using a modified stir-fry method than in microwave and conventional cooking procedures. Eheart (1967) found that in broccoli, more chlorophyll was lost during cooking than in microwave heating or water blanching followed by 12 months storage at $0^{\circ}F$.

Sweeney and Martin (1958,1961) examined the effect of preparation and cooking methods on chlorophyll stability in frozen broccoli, brussels sprouts, green beans, lima beans, green peas and spinach. They found

that chlorophyll retention during cooking was related to the pH of the vegetables, those with a high pH giving the better pigment retention. Cooking in buffers of pH6.2-7.0, also gave better colour retention at the higher levels of pH.

2.2215 Kinetics of Chlorophyll Degradation

The kinetics of chlorophyll degradation in heat processed and cooked foods has been reported by a number of workers. Gold and Weckel (1959) stated that their results on processed peas were best represented by pseudo first-order kinetics. Comparison of rates of degradation for chlorophyll a and chlorophyll b in spinach (Tan and Francis 1962; Gupte 1963; Clydesdale 1966), broccoli (Sweeney and Martin 1958; Eheart 1967) and other vegetables (Sweeney and Martin 1961) have shown that chlorophyll a is degraded more rapidly than chlorophyll b during blanching, heat processing, cooking or storage, although not to the same extent as found in in vitro systems containing pure pigments (Schanderl et al.1962). Chlorophyllide a was also found to be degraded more rapidly than chlorophyllide b in H.T.S.T. processed spinach puree (Clydesdale 1966), in agreement with the results of Schanderl et al. (1962).

2.222 Freezing

Colour changes in stored frozen plant material may be caused enzymically as well as non-enzymically for it is widely recognised that enzymic activity is not inhibited by storage at the temperatures normally used in frozen food distribution i.e. -10°F to 10°F. Numerous enzymes have been shown to maintain some activity at these temperatures leading to deterioration of colour and flavour on storage, and consequently a

heat treatment is employed to inactivate enzymes since much lower temperatures e.g. -50°F, are economically not feasible. At the present time, the value of blanching green vegetables prior to freezing and storage is almost universally recognised and accepted as a means of preserving colour and quality. The effect of blanching on the colour and/or pigments of frozen vegetables has been examined by a number of workers (Diehl and Berry 1933; Dietrich et al.1955,1959b; Van Buren et al.1964; Walker 1964; Dietrich and Neumann 1965; Last and Shipton 1966). Results have shown that, in general, blanching designed to achieve the inactivation of peroxidase gives products of acceptable quality after freezing and storage as judged by colour and pigment retention. The time-temperature combination for blanching depended on the size, shape and consistency of the material concerned.

Van Buren et al.(1964) showed that the conversion of chlorophyll to pheophytin in beans was more rapid following a 60-80°C blanch than after no blanch or a 100°C blanch, indicating that a heat-activated factor, possibly the enzyme pectin methyl esterase, was necessary to initiate the conversion and oxidation of chlorophylls. A decrease in pH and an increase in alcohol-insoluble acid groups found during post-blanch storage of snap beans was in agreement with such a hypothesis.

Walker (1964b) studied the effect of blanching treatments on the colour deterioration and pigment degradation of frozen French beans and found conversion of chlorophylls to pheophytins. During subsequent storage at -10°C, however, both conversion and oxidation occurred, and he concluded that a blanch time, of the order of 45-60 seconds, should

be selected such that enzymes are inactivated without undue heat initiation of systems which may result in chlorophyll oxidation. Under these conditions, colour deterioration in frozen storage could be measured by conversion of chlorophyll to pheophytin, but with blanch times shorter or longer than those recommenced, chlorophyll oxidation would become an important factor in the overall assessment of French bean colour.

With frozen vegetables, one of the important functions of blanching is the inactivation of enzymes such as peroxidases, lipases and lipoxygenases. The action of such enzymes results in colour changes associated with chlorophyll conversion and destruction (Wagenknecht et al.1952; Wagenknecht and Lee 1956; Walker 1964a,b) and the development of disagreeable off-flavours in the lipid fraction (Lee and Wagenknecht 1951; Lee 1954; Lee et al.1955,1956; Wagenknecht and Lee 1958; Whitfield and Shipton 1966; Grosch 1967).

Many reports in the literature have discussed the effects of maturity, storage temperature and other factors on the stability of colour and chlorophyll pigments in frozen vegetables. In particular, attention has been directed to peas (Lindquist et al.1950; Eastmond et al. 1951; Boggs and Talburt 1952; Dietrich et al. 1955,1957a,b; Boggs et al. 1960; Pinsent et al.1962; Last and Shipton 1966), beans (Dietrich et al. 1959a,b; Sweeney et al.1961), broccoli (Sweeney and Martin 1958), brussels sprouts (Dietrich and Neumann 1965) and spinach (Dietrich et al. 1960). The time-temperature tolerance (T.T.T.) of frozen foods has been closely examined by Dietrich and coworkers at the Western Regional Research Laboratory, U.S.A.. These workers were able to show that long

term storage stability of quality characteristics, including colour and pigments, depended not only on storage time and temperature, but also on storage temperature fluctuations and the previous history and nature of the material.

2.3 SEPARATION AND ESTIMATION OF CHLOROPHYLL PIGMENTS

2.31 Separation of Chlorophylls and Their Derivatives

The separation of chloroplast pigments extracted from plant materials has been extensively investigated by a number of different methods. Early work on the separation of chlorophylls and their derivatives was usually based on differential solubility in immiscible solvents, but since the introduction of rapid chromatographic procedures for pigment analysis, these methods have largely been replaced. Various aspects of the separation and analysis of chlorophylls have been reviewed by Smith and Benitez (1955), Sestak (1958,1965), Egle (1960), Holden (1965b), Strain and Svec (1966) and Strain (1968).

Most methods of separation and determination of chloroplast pigments depend upon an initial extraction procedure to remove the pigments from the plant material. The choice of solvent used depends on the nature of the plant material and whether the extract is to be chromatographed or used for pigment estimation. The most common solvents are acetone, methanol or methanol-petroleum ether mixtures. The nature and properties of various solvents have been discussed by Holden (1965b) and Strain and Svec (1966).

The basis of most methods used for the isolation and characterisation of chloroplast pigments was the discovery, by Tswett (1906),

that filtration of a plant extract through columns of finely powdered calcium carbonate, inulin or powdered sugar resulted in the resolution of the extract into several coloured zones. He was able to show that plant extracts resolved into two green pigments, now known as chlorophylls a and b, as well as yellow carotenoid pigments. These early experimental methods were elaborated and improved by Willstatter and coworkers (Willstatter and Stoll 1913) and others, but the separations obtained were still incomplete. Zscheile (1934a,b) prepared pure chlorophylls a and b by adsorption on talc for the final purification step, and measured quantitatively the adsorption spectra of the pure components by means of a photoelectric spectrophotometer.

When these early separations showed that pure pigments could be obtained, a large number of papers appeared reporting the column separation of chlorophyll pigments, describing many different types of adsorbent, solvent systems and elution behaviour for the chlorophylls and their degradation products. The most widely used adsorbent for column chromatography has been powdered icing sugar, usually containing up to 5% starch to prevent caking. The chromatographic sequence of chlorophylls a and b and their derivatives on powdered sugar columns, often used as a basis for their description and identification, is given in Table 2.4 (Strain and Svec 1966; Strain 1967).

Table 2.4 Chromatographic Sequence of Chlorophylls a and b and Some of Their Alteration Products in Columns of Powdered Sugar and Petroleum Ether Plus 0.5% - 2% n-propanol as the Wash Liquid

(from Strain and Svec 1966)

	Pyrochlorophyll b	(most adsorbed)
	Chlorophyll b	
incompletely separated	Chlorophyll b'	
incompletely separated	Methyl pyrochlorophyllide a	
	Methyl chlorophyllide a	
	Methyl chlorophyllide a'	
	Methyl pheophorbide a	
	Methyl pyropheophorbide a	
	Pyrochlorophyll a	
	Chlorophyll a	
	Chlorophyll a'	
	Pheophytin a	
	Pyropheophytin a	(least adsorbed)

Other adsorbents and adsorbent mixtures, which may alter the separation and sequence of pigments, have been used as alternatives to powdered sugar. These include cellulose powder, starch, 70:30 sugar-starch mixtures, 6:1 sugar-talc, paper pulp, ion-exchange resins, magnesium citrate, inulin, and polyethylene powder (Holden 1965b; Strain and Svec 1966).

Until the time Smith and Benitez (1955) wrote their review, few papers had appeared on the paper chromatography of chlorophylls. In the intervening years, this method has received extensive consideration and investigation. Reports in the literature on the separation of chlorophylls and their derivatives have described various papers, solvent systems and methods of development (Sestak 1958,1965; Strain and Svec 1966; Strain

1967). These include one-way, two-way and radial chromatography, with or without centrifugal force acceleration. Various modifications of such techniques may be used for specialised applications. Reports have shown that paper chromatography can separate up to 8 chlorophyll-type pigments (Hager 1957; Holden 1962).

Although thin-layer chromatography was introduced only recently, its potential has been widely recognised in pigment research. The method has become a powerful tool for the pigment chemist because of its capacity to quickly separate large numbers of structurally similar pigments using inexpensive equipment. Its use in the separation of chloroplast pigments, including chlorophylls a and b and their breakdown products, has been demonstrated by many workers using a variety of different techniques. Techniques differ in the adsorbent used, the method of plate preparation, the thickness of the layer, the developing solvent, the atmosphere inside the tank, and other respects. Some of the adsorbents used for chloroplast pigments include silica gel with or without ascorbic acid, cellulose powder, kieselguhr, and sugars such as sucrose, glucose and mannitol.

Recent reports by Bacon (1966,1967), Bacon and Holden (1967a,b) and Strain et al.(1967) have thrown some doubts on the value of silica gel T.L.C. for the separation of large numbers of chlorophyll derivatives present in pigment extracts obtained from various sources. Schanderl and Lynn (1966) and Lynn and Schanderl (1967a,b) reported the separation on silica gel G plates of up to 16 pigment spots in a two-dimensional development of pure pigments and extracts of ripening peppers, banana peels and cucumbers. They reported the presence of pigments with unusual

absorption spectra and chromatographic behaviour, and proposed that their resolution was due to the superior resolving power of this adsorbment under the conditions used. The formation of artifacts was ruled out by subsequent experiments, and it was claimed that these compounds were initially present in the pigment extracts. Working with thin layers of cellulose, Bacon (1966,1967) showed that pigments, with spectra and properties almost identical to those obtained by Schanderl and Lynn, were produced as artifacts when pure solutions of chlorophyll pigments were chromatographed or adsorbed on silica gel layers under the conditions described by Schanderl and Lynn. The artifacts were similar to pigments isolated by Michel-Wolwertz and Sironval (1965) by paper chromatography of extracts from Chlorella. Strain et al.(1967) also reported that several chloroplast pigments, including chlorophylls, were altered when extracts were chromatographed on siliceous adsorbents.

Subsequent reports by Bacon and Holden (1967a,b) indicated that these unusual pigments were identical to "changed" chlorophylls produced by the action of organic solvents or alkaline solutions on pure pigments or the pigments of boiled leaves. The available evidence indicated that "changed" chlorophylls a-1, a-2 and a-3, and "changed" chlorophylls b-1, b-2 and b-3, the names given to the alteration products described by Bacon and Holden (1967a,b), were in fact oxidation products of the parent chlorophylls a and b, possibly 10-hydroxychlorophylls and/or lactone derivatives (Pennington et al.1967). From work such as this, it is apparent that while thin-layer chromatography is capable of greater resolution than some other forms of chromatography, great care must be

taken to ensure that artifacts are not produced during the separations, particularly with labile materials such as the chlorophylls.

2.32 Estimation of Chlorophylls

A large number of chemical compounds in solution may be characterised and identified by their spectral absorption properties. The chlorophyll pigments, because of their porphyrin structure, highly conjugated system of double bonds and characteristic groups, show well-defined absorption spectra in different solvents over both the ultraviolet (UV)-visible (approx. 200-700nm) and infrared (approx. 2,000-16,000nm) ranges.

2.321 Absorption Spectra

2.3211 UV-visible

Early work on the spectral curves of extracted chlorophylls did not agree very well either in the position of the absorption maxima or in the values of the extinction coefficients. Improved chromatographic techniques have now enabled several workers to obtain chlorophylls that meet high standards of spectroscopic purity and reproducibility. Zscheile and Comar (1941) studied the influence of the preparative procedure on the visible absorption spectra of chlorophylls a and b. They described a method of preparing chlorophyll solutions with reproducible spectroscopic properties and emphasised that pigment drying and delays in spectral observations should be avoided.

Mackinney (1941) reported that the solvent had an important effect on the absorption coefficients of chlorophylls a and b. Later investigations (Harris and Zscheile 1943) compared 13 solvents with chlorophyll a and 5 solvents with chlorophyll b after direct elution from sucrose

adsorption columns. More recently, Seely and Jensen (1965) examined the visible spectrum of chlorophyll a in forty solvents covering a wide range of dielectric constant and refractive index, and found various correlations between solvent type and spectral properties.

The quantitative absorption curve of chlorophylls a and b have been measured in a number of different solvents. Wavelengths of maximum absorption and specific absorption coefficients for chlorophylls a and b and pheophytins a and b in diethyl ether and acetone are summarised in Table 2.5.

Table 2.5 Absorption Maxima and Specific Absorption Coefficients of Chlorophyll Pigments in Diethyl Ether and Acetone

Pigment	Solvent	Absorption Maxima (λ,nm) and Specific Absorption Coefficients (α,l/g.cm.)	Reference
Chlorophyll a	Diethyl Ether	410, 429, 532, 576, 614, 660 λ 85.2,135.0,4.14,8.51,15.5,102.1 α	Zscheile and Comar (1941)
		661 102.0	Davidson (1954)
		410, 430,535.5,578, 615, 662 85.2,131.5,4.22,9.27,16.3,100.9	Smith and Benitez (1955)
		428.5 660.5 125.1 96.6	Strain et al. (1963)
		428.8 660.6 95.2	Seely and Jensen (1965)
		428.9 662 154.2 112.7	Brown (1968)
	Acetone	410, 430, 535, 580, 615, 663 77.1,106.0,3.88,8.67,15.7, 84.0	Mackinney (1940)
		663 92.6	Vernon (1960)
		430.1 662.0 85.7	Seely and Jensen (1965)
	80% Acetone	433, 536, 582, 618, 665 101.5,4.78,11.6,19.6, 90.8	Vernon (1960)

Table 2.5 (Contd.)

Pigment	Solvent	Absorption Maxima (λ,nm) and Specific Absorption Coefficients (α,l/g.cm.)	Reference
Chloro-phyll b	Diethyl Ether	430, 453, 550, 594, 642.5 λ 58.5, 171, 6.2, 10.9, 56.8 α	Zscheile and Comar (1941)
		644 62.0	Davidson (1954)
		430, 455, 549, 595, 644 62.7, 174.8, 7.07, 12.7, 62.0	Smith and Benitez (1955)
		452.5 647 175.3 61.8	Strain et al. (1963)
		453 642.5 221.4 79.9	Brown (1968)
	Acetone	455, 595, 645 146.9 11.3, 51.8	Mackinney (1940)
		647 53.5	Vernon (1960)
	80%	460 600, 648-649 148 14.3, 52.5	Vernon (1960)
Pheo-phytin a	Diethyl Ether	410, 470, 505, 532, 559 608, 665 126.0, 4.5, 13.5, 11.3, 3.2, 8.8, 59.0	Zscheile and Comar (1941)
		667 65.0	Davidson (1954)
		408.5, 471, 505, 534, 560, 609.5, 667 132, 5.1, 14.6, 12.6, 3.6, 9.8, 63.7	Smith and Benitez (1955)
		408, 668 155.6, 76.7	Brown (1968)
	80% Acetone	409, 472, 505, 536, 610, 666-667 130.9, 6.3, 15.0, 13.1, 11.9, 56.6	Vernon (1960)
Pheo-phytin b	Diethyl Ether	413, 433, 523, 558, 599, 653 77, 197, 12.6, 7.5, 8.1, 37.0	Zscheile and Comar (1941)
		655 41.8	Davidson (1954)
		412.5, 434, 525.5, 555, 599, 655 83.0, 216, 14.2, 8.7, 9.5, 42.1	Smith and Benitez (1955)
	80% Acetone	436, 527 655 181, 14.9 35.7	Vernon (1960)

The specific absorption coefficient (α_λ) at wavelength λ(nm) is defined as

$$\alpha_\lambda = \frac{A}{dc}$$

where A = absorbance or optical density
d = length of light path (cm)
c = concentration of pigment (g/litre).

A convenient test for the presence of pheophytin in ether solutions of purified chlorophyll was devised by Zscheile and Comar (1941). They defined the purity ratio, R_a, for chlorophyll a as the absorption at 660nm divided by the absorption at 505nm, the latter wavelength being a minor peak of pheophytin a and a point of minimum absorption in the chlorophyll a spectrum. Pure chlorophyll a gave a ratio of 54, but a lower value was found if pheophytin a was present. For chlorophyll b, the purity ratio, R_b, was taken as the absorption at 642.5nm divided by that at 520nm. Its maximum value for a pure preparation was 18.9.

The ratio of the heights of the major "blue" and "red" absorption bands for chlorophylls a and b has also been reported by many workers as a useful criterion of purity (Table 2.6). Aronoff (1962) and Anderson and Calvin (1962) claimed that purification of chlorophyll a by chromatography on columns of powdered polyethylene resulted in a product with higher purity than that prepared by sugar chromatography, and reported lower ratios of "blue" (428-430nm) to "red" (660nm) absorbances (Table 2.6). They attributed the higher ratios of other preparations to the presence of blue-absorbing carotenoids. Subsequent work by Perkins and Roberts (1964) using ^{14}C tracer studies showed that the limiting blue/red ratio for chlorophyll a was 1.29, with average figures in the range 1.29-1.33. The lower ratios found by Aronoff (1962) and Anderson and Calvin

(1962) were shown to be due to the presence of water, alcohols or other impurities in the diethyl ether used for spectroscopy.

Table 2.6 Absorption Ratio $\frac{\text{blue}}{\text{red}}$ for Chlorophylls a and b in Diethyl Ether

Reference	Chlorophyll a	Chlorophyll b
Zscheile and Comar (1941)	1.32	2.82
Harris and Zscheile (1943)	1.33	2.98
Holt and Jacobs (1954)	1.33	-
Smith and Benitez (1955)	1.30	2.82
Aronoff (1962)	1.17	-
Anderson and Calvin (1962)	1.19	-
Strain et al. (1963)	1.30	2.84
Perkins and Roberts (1964)	1.33	-
Seely and Jensen (1965)	1.31	-

The absorption spectra of crystalline ethyl chlorophyllides a and b in diethyl ether were shown by Holt and Jacobs (1954) to be almost identical to the spectra of the parent chlorophylls a and b as determined by Zscheile and Comar (1941), showing that the length of the side chain had little, if any, influence on the visible spectrum. Similarly, the spectra of ethyl pheophorbides a and b were almost identical to the parent pheophytins a and b. These findings have since been verified by Schanderl et al.(1962) for ethyl, methyl and free chlorophyllides a and b.

2.3212 Infrared and N.M.R.

In recent years, the use of infrared and nuclear magnetic resonance (N.M.R.) spectroscopy has been extensively studied for the determination of structure and configuration of chlorophyll pigments. However, these techniques are not applicable to the quantitative estimation of chloro-

phylls, and will not be discussed in this work. The subject has been thoroughly reviewed by Katz et al. (1966).

2.322 Estimation by Spectrophotometry

The importance of chlorophyll pigments in determining the colour of green plant foods of interest to the food technologist, has necessitated the development of analytical procedures for their qualitative and quantitative determination. This involves the complete extraction of the pigments in known form, and a reliable, accurate measurement of the pigments in the extract. As an alternative to quantitative chromatographic separation and estimation of the individual pigments, which is often difficult to achieve, considerable attention has been focussed on colorimetry and spectrophotometry as rapid, reproducible methods for the estimation of chlorophylls. Analysis of the "red" region of the visible spectrum, from 640nm to 700nm where carotenoid pigments do not absorb, enables the chlorophylls and their breakdown products to be estimated. Reviews on chlorophyll determinations have been published by Smith and Benitez (1955), Holden (1965b), Ziegler and Egle (1965), and Strain and Svec (1966).

2.3221 Chlorophylls a and b and Total Chlorophyll

The spectrophotometric determination of chlorophylls a and b in a mixture of pigments is dependent on the Lambert-Beer Law. For any wavelength λ,

$$\text{Absorbance} = A_\lambda = \alpha_a C_a d + \alpha_b C_b d = \alpha_a C_a + \alpha_b C_b$$

for solutions of chlorophylls a and b in a 1cm cuvette, where α_a and α_b are the specific absorption coefficients of C_a and C_b at wavelength λ,

and c_a and c_b are the concentrations of the pigments in g/litre. If absorbances at two different wavelengths are measured, the two equations can be solved simultaneously for c_a and c_b.

Arnon (1949) used the specific absorption coefficients for chlorophylls a and b in 80% acetone established by Mackinney (1941) to set up two equations for the estimation of chlorophylls a and b and total chlorophyll. Absorbances were measured at 663nm and 645nm, the absorption maxima for chlorophylls a and b in 80% acetone. Bruinsma (1963) showed that the quantitative absorption curves of chlorophylls a and b in 80% acetone intersected at 652nm where the specific absorption coefficient was 36.0, and from these figures, derived the expression $27.8A_{652}$ for the determination of total chlorophyll in mg/litre.

The turbidity often associated with aqueous acetone extracts of plant material can be eliminated by transferring the pigments to diethyl ether in which the spectrophotometric measurements are then made. Comar and Zscheile (1942) reported equations for the estimation of chlorophylls in diethyl ether, which were later adopted as the official method of chlorophyll analysis in plants by the A.O.A.C. (1960). Absorbances were measured at 660nm and 642.5nm. Smith and Benitez (1955), used their own determinations of wavelength maxima and specific absorption coefficients (Table 2.5), and obtained slightly different equations for chlorophyll determination. A check on total chlorophyll concentration using the expression $100.5A_{600}$ was made since the spectra of chlorophyll a and b in diethyl ether intersected at 600nm with a specific adsorption coefficient of 9.95.

A critical examination of various aspects of the spectrophotometric determination of chlorophylls a and b in acetone and diethyl ether was made by Ziegler and Egle (1965). These workers examined the effects on the errors of determination of extraction, acetone concentration, instrument wavelength calibration, temperature and absorbance correction at 750nm. They derived sets of equations for 80% acetone and diethyl ether based on experimental results.

A new method estimating the concentration of chlorophylls a and b accurately and sensitively was devised by Ogawa and Shibata (1965). The method was based on the principle that hydroxylamine reacted with the aldehyde group of chlorophyll b in 95% methanol at pH5.8 to give a derivative having a red absorption similar in position to that of chlorophyll a, while chlorophyll a remained unaffected. Pigment concentrations were determined by measuring absorbances at a single wavelength, 666nm, before and after the addition of hydroxylamine.

Sestak (1966a) has recently described the construction of a simple nomogram for the determination of chlorophylls a and b, total chlorophyll and pigment a/b ratio. The nomogram was used instead of a set of two equations in a two-wavelength spectrophotometric pigment determination.

2.3222 Chlorophylls, Pheophytins and Their Derivatives

The processing and storage of foods containing chlorophyll pigments results in the formation of breakdown products such as pheophytins, chlorophyllides and pheophorbides. Methods for the estimation of chlorophylls a and b will necessarily produce large errors when applied to extracts containing pheophytins and other breakdown products since the

latter pigments also absorb in the "red" region of the visible spectrum but give different spectra to the parent chlorophylls.

The determination of the percent conversion of chlorophyll to pheophytin was examined in aqueous acetone solutions by Mackinney and Weast (1940) and Dietrich (1958) and in acetone by Sweeney and Martin (1958). The method of Mackinney and Weast (1940) was based on the observation that the maximum increase in absorbance occurred at 535nm when 90% acetone extracts of frozen vegetables were treated with oxalic acid solution, while there was no change at 560nm, the crossover or isobestic point of chlorophyll and pheophytin spectra. Subsequent re-evaluation by Dietrich (1958) has placed these points at 534nm and 556nm for 80% acetone extracts. Percent conversion of chlorophyll to pheophytin, treating the pigments as a two-component system, was determined by the equation: $\text{Percent Conversion} = \frac{R_x - R_o}{R_{100} - R_o} \times 100$

where R_o = absorbance ratio $\frac{A_{534}}{A_{556}}$ for zero conversion, R_{100} = absorbance ratio $\frac{A_{534}}{A_{556}}$ for 100% conversion, and R_x = absorbance ratio $\frac{A_{534}}{A_{556}}$ for unknown extract. Sweeney and Martin (1958) modified these methods and calculated percent retention of chlorophylls in acetone extracts of cooked broccoli. Absorbance changes were recorded at 535nm, 642nm and 665nm after conversion of chlorophylls to pheophytins with oxalic acid.

Reflectance spectrophotometry was used by Kapsalis et al.(1965) to quantitatively follow the destruction of green pigment in heat processed peas. They found that a close correlation ($r = 0.95$) existed between percent conversion of chlorophyll to pheophytin (Dietrich 1958) and the empirical ratio $(As_{632nm} - As_{536nm})/As_{582nm}$, or "chlorophyll stability

index", where As=2 - log percent reflectance against the standard, the latter being used to establish the 100% reflectance line.

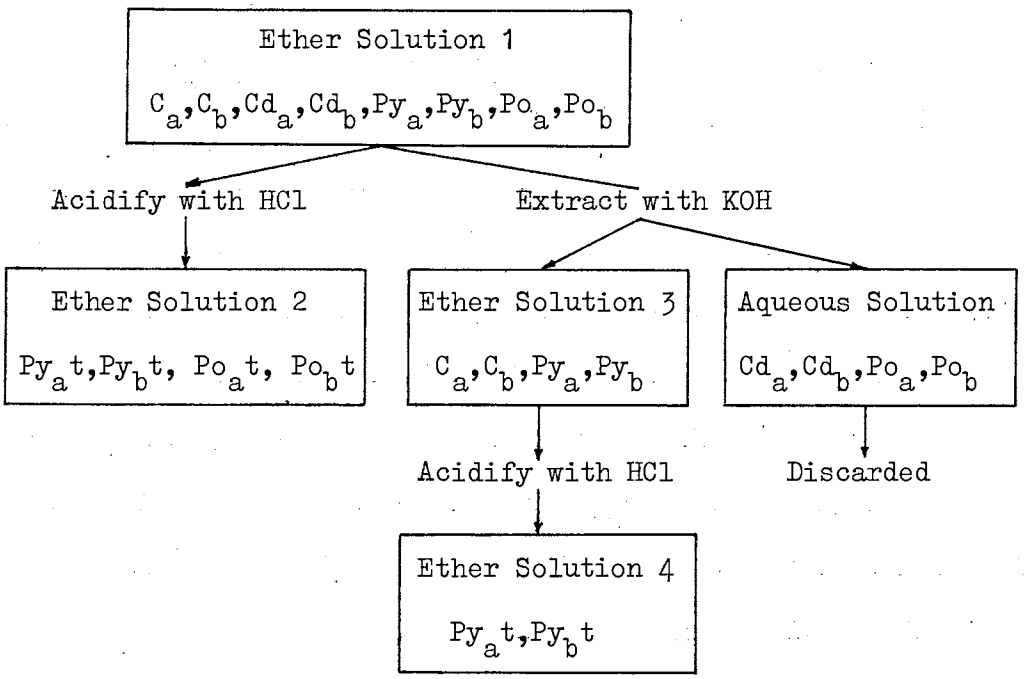
Vernon (1960) modified the method of Sweeney and Martin (1958) to enable total chlorophyll, total pheophytin, chlorophylls a and b, pheophytins a and b, and percent retention of chlorophyll to be determined in 80% acetone extracts of green vegetables. Treating the extracts as four-component systems, Vernon derived a series of equations for pigment estimations based on absorbance changes at various wavelengths before and after addition of oxalic acid (Section 4, Expt. 7.3). Concentrations of chlorophylls a and b and total chlorophyll were determined from absorbance readings at 662nm and 645nm, while concentrations of pheophytins a and b were determined with two sets of equations from readings at 666nm, 655nm and 536nm, the latter being a minor absorption peak in the pheophytin a spectrum. Pigment concentrations calculated using 655nm readings were found to be less accurate than those using 536nm readings, since the former were on the steep part of the curve. Comparison of the results for percent chlorophyll retention with those obtained by the methods of Dietrich (1958) and Sweeney and Martin (1958) showed that the latter two procedures gave consistently higher results. This discrepancy was probably due to their calculations being based on a two-component, instead of a four-component, system.

Pigment concentrations in acetone solutions extracted from heat processed spinach puree were determined by Tan and Francis (1962) after chromatographic separation of the pigments on sugar-starch columns. Equations reported by Comar and Zscheile (1942) for ether solutions were

modified for use in acetone solutions, and chlorophylls a and b were eluted together and determined from equations using absorbance readings at 660nm and 642.5nm respectively. Pheophytins a and b were eluted separately from the column and estimated from absorbance readings at 667.5nm and 655nm respectively.

Equations have also been developed for estimation of several pigments in diethyl ether solutions (Davidson 1954; Jones et al.1961,1962; White et al.1963). The isolation of pheophorbides from brined cucumbers by Jones et al.(1962) led these workers to develop a method for the estimation of six pigments, chlorophylls a and b, pheophytins a and b, and pheophorbides a and b in the one ether extract. This work on pigment changes in brined cucumbers was carried further by White et al.(1963), who reported the successful use of a method in which chlorophyllides a and b, in addition to the other six pigments, could be estimated. The scheme for the estimation is shown in Figure 2.3. Absorbances were measured at 660nm and 642.5nm for ether solutions 1 and 3, and at 666.5nm and 653nm for ether solutions 2 and 4. They assumed that the absorption coefficients of the chlorophyllides and pheophorbides did not differ significantly from those of the respective chlorophylls and pheophytins, an assumption that has been shown by Holt and Jacobs (1954) to introduce little error. Absorption coefficients reported previously by Zscheile and Comar (1941) and Holt and Jacobs (1954) were used to establish a series of 16 equations for pigment estimation (Section 4, Expt. 6.1). This method was subsequently used by Jones et al.(1963) for pigment determinations on blanched and brined vegetables, and by Clydesdale (1966) for pigment changes in H.T.S.T. processed spinach puree.

Figure 2.3 Scheme for Estimation of Chlorophylls and Derivatives in Diethyl Ether (White et al.1963)



Walker (1964a) calculated concentrations of chlorophylls a and b and the respective pheophytins in frozen French beans by equations derived by matrix inversion of data from Aronoff (1953) using absorbances measured at 660nm, 642.5nm, 525nm and 505nm in absolute ethanol. Wickliff and Aronoff (1962) determined chlorophylls in 90% aqueous ethanol extracts of plant tissues after conversion into the pheophytins with hydrochloric acid. A similar technique was used by Wilson and Nutting (1963), although these workers removed magnesium from chlorophylls on a column of ion-exchange resin in the acid form, at the same time separating the resulting pheophytins. Material that could cause breakdown of pheophytins was also removed, and the pheophytins obtained were claimed to be more stable than those made by conversion with acids. This technique, however, did not allow estimation of percent conversion of chlorophylls,

but only the amount of a, b and total pigments as either chlorophyll or pheophytin.

The resin separation method of pigment estimation was compared (Nutting and Becker 1966) to results obtained from Vernon's (1960) techniques of measuring a, b and total pigments as chlorophylls or as pheophytins. Each of these three procedures proved equally good, depending on the product analysed, the time available, and whether the results were desired as total chlorophyll or a/b ratios. Eheart and Gott (1965) also compared methods for chlorophyll analysis in raw and cooked broccoli reported by Sweeney and Martin (1958), A.O.A.C. (1960) and Vernon (1960). Correlation coefficients showed good agreement between A.O.A.C. and Vernon equations for total chlorophyll from raw broccoli ($r = 0.98$), but a poor correlation for chlorophyll a in cooked broccoli ($r = 0.39$). The correlation for chlorophyll b in cooked broccoli was much higher ($r = 0.83$). The study showed that the A.O.A.C. (1960) method gave satisfactory results for raw broccoli, but not for cooked broccoli, while the Sweeney and Martin equations gave results similar to the Vernon equations for total chlorophyll concentration ($r = 0.97$), but somewhat higher results for percent chlorophyll retention.

2.323 Other Methods

Although spectrophotometric methods of chlorophyll determination are by far the most widely used, other techniques have been reported in the literature. These include fluorimetry, magnesium analysis (for chlorophyll pigments only), and non-destructive methods for measuring the chlorophyll content of leaves. These subjects have been adequately

reviewed by Smith and Benitez (1955) and Holden (1965b). Inada (1963, 1965) has recently described the application of a photoelectric "chlorophyllometer" for estimating the chlorophyll content of intact leaves. In most cases studied, high correlation coefficients were found between the meter readings and chlorophyll concentrations determined by normal spectrophotometric procedures in 80% acetone.

2.4 COLOUR OF CHLOROPHYLL-CONTAINING FOODS

Colour has long been recognised as one of the most important quality attributes of foods. The first impression of a food is usually visual and a major part of a consumer's willingness to accept a food depends on its colour. Colour can serve as a useful criterion of quality, and in many cases is an indicator of various types of deteriorative changes undergone by the food. The extent to which a consumer will allow variation in the colour of a given food depends on a preconceived idea of what he believes the food colour should be, and, to a lesser extent, which colour is preferred. Foods containing chlorophyll pigments are particularly susceptible to consumer discrimination, because of the large variation in colour that can appear between fresh, frozen and heat processed materials. Interest in food colour, its measurement and specification, has developed because of its close correlation with consumer acceptability, and has resulted in attempts to objectively define the colours of and colour differences between various foods. This is particularly so in relation to quality grading of agricultural products.

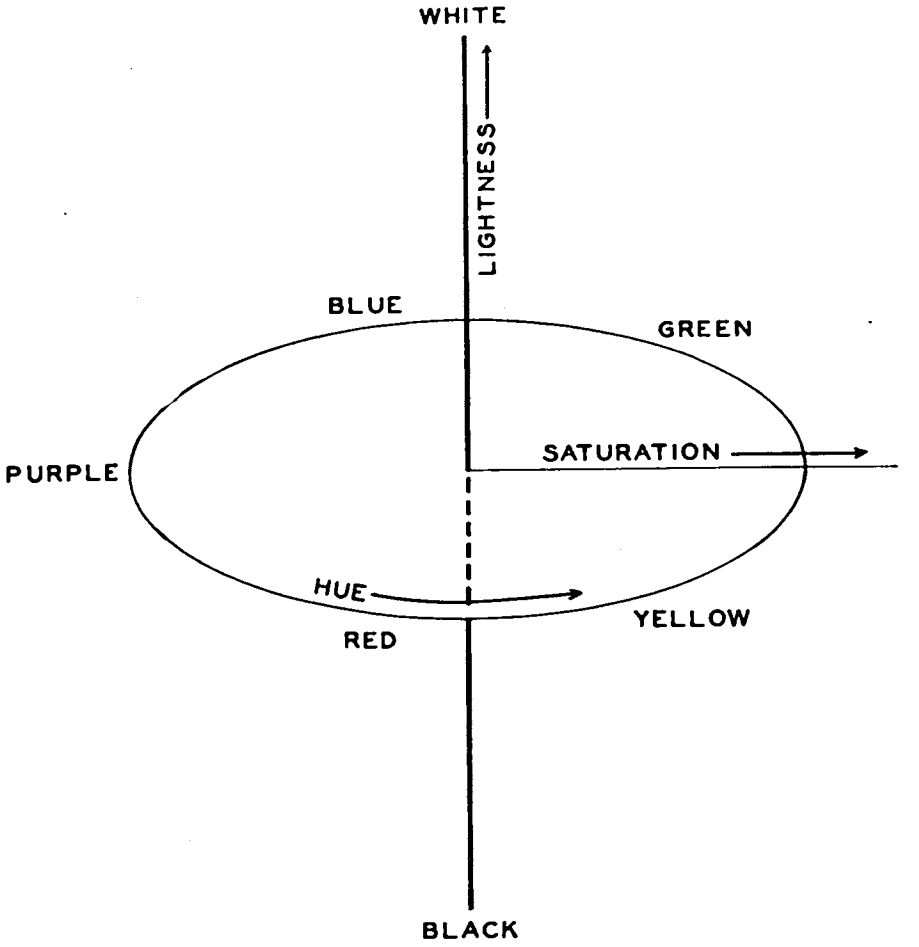
2.41 Psychophysical Aspects of Colour

Various aspects of the physics and psychology of colour and colour measurement have been discussed in a number of detailed reports including

those of Hardy (1936), Nickerson (1946), Bouma (1947), Evans (1948), Judd (1950), Committee on Colorimetry, Optical Society of America (1944, 1953), Burnham et al.(1963), Judd and Wyszecki (1963), Wright (1964) and Billmeyer and Saltzman (1966).

The definition of colour most widely accepted is that reported by the Committee on Colorimetry of the Optical Society of America (1944): "Colour consists of the characteristics of light other than spatial and temporal inhomogeneities; light being that aspect of radiant energy of which a human observer is aware through the visual sensations which arise from the stimulation of the retina of the eye". Colour defined in this way is thus a psychophysical concept in that it is identified with both (physical) radiant energy as well as with (psychological) visual sensation. The characteristics of light which give rise to colour sensations can be specified in terms of the appropriate photometric quantity, the dominant wavelength and the purity. These three characteristics of light approximate to the attributes of visual sensations known of as lightness, hue and saturation. It was recognised early that complete specification of colour, as seen by human observers, required three dimensions which are used to describe points plotted in a space diagram or surface-colour solid (Figure 2.4). Lightness is the attribute associated with brightness of the colour, or the extent to which a hue is diluted with white, grey or black. Hue is the attribute associated with the kind of colour, whether it is red, green, etc. Saturation is the attribute associated with the depth or strength of the colour, and is a measure of the degree of difference from a grey of the same lightness.

The chromatic aspects of colour, hue and saturation, are referred



(from Judd 1950)

FIGURE 2.4

Dimensions of the psychological surface-colour solid.

to as chromaticity, while white, grey and black, the achromatic aspects, determine only the lightness of a colour. It is necessary to relate these dimensions of colour to properties of the light which stimulates the retina in the eye. The amounts of light in different parts of the visible spectrum (usually referred to as wavelengths between 400nm and 700nm) reflected or transmitted by a coloured body can be measured by a spectrophotometer. A spectral curve showing intensity of absorption or reflection and wavelength will hence give a complete specification of colour in physical terms.

It has been shown that any colour can be matched exactly by a suitable mixture of three colours, called primaries, selected such that no single primary can be matched by any mixture of the other two. Methods developed for the specification of colours describing lightness, hue and saturation in terms of tristimulus readings obtained under standard conditions have been embodied in the C.I.E. and Munsell systems of colour notation.

2.42 Systems of Colour Notation

2.421 C.I.E. System

An internationally accepted system of colour standardisation has been established by the Commission Internationale de l'Eclairage (C.I.E.) (1931). The C.I.E. system brings together the physical and psychological aspects of colour by defining three theoretical primary colours or reference stimuli, a standard observer, standard illuminants, and a standard system of co-ordinates. The three primaries, designated (X), (Y) and (Z), were chosen so that a mixture of equal amounts of each

matched a standard white. Since it was considered desirable to ensure that all real colours could be specified entirely in terms of positive quantities, this required that the three reference stimuli should be imaginary, rather than real colours. The imaginary primaries (X), (Y) and (Z) were related to a set of real primaries, R, G and B of wavelengths 700nm, 546.1nm and 435.8nm respectively by the following relationship (Mackinney and Little 1962):

$$R = a_1(X) + b_1(Y) + c_1(Z)$$

$$G = a_2(X) + b_2(Y) + c_2(Z)$$

$$B = a_3(X) + b_3(Y) + c_3(Z)$$

As an additional mathematical device, which greatly simplified calculations, two of the reference stimuli (X) and (Z) were made to have zero luminous efficiency i.e. all of the light energy represented by a colour was regarded as coming from the stimulus (Y). Thus the amount of (Y) was then a direct measure of the lightness dimension of the colour.

Under the C.I.E. system, an equation can be written for any real colour (C) as seen by a standard observer under standard conditions. Thus: $C(C) = X(X) + Y(Y) + Z(Z)$. The amounts X, Y and Z of the three standard primaries (X), (Y) and (Z), required to match an amount C of the colour (C) are termed the tristimulus values of the colour. Making C equal to a trichromatic unit of colour, $1(C) = x(X) + y(Y) + z(Z)$. The tristimulus values X, Y and Z can hence be expressed more conveniently in terms of the chromaticity co-ordinates x, y and z, where:

$$x = \frac{X}{X+Y+Z}, \quad y = \frac{Y}{X+Y+Z}, \quad z = \frac{Z}{X+Y+Z} \quad \text{and} \quad x+y+z = 1$$

The two ratios x and y serve to specify the chromatic aspects of the light. Any colour can therefore be specified in terms of the C.I.E. co-ordinates by x, y, defining the chromatic aspects, and by Y, defining the achromatic aspects.

When C.I.E. values x and y for all real colours are plotted on rectangular co-ordinates, all of the points fall within the limits of a plane figure called a chromaticity diagram (Figure 2.5). The spectrum locus in this diagram is the line of separation between real and unreal (imaginary) colours. If Y, the lightness dimension of a colour, is plotted perpendicularly to the chromaticity plane, an irregular colour solid is created within which any real colour can be fixed as a unique point by C.I.E. co-ordinates x, y and Y.

Three sources or illuminants A, B and C were standardised by the C.I.E.. Source A was representative of gas-filled incandescent lamps; source B was representative of noon sunlight; and source C was representative of average daylight such as that from a completely overcast sky. The most commonly used, illuminant C, was specified by a lamp of colour temperature about $6770^{\circ}K$ in conjunction with a two-cell filter.

The standard observer in the C.I.E. system was defined by the tristimulus values of the spectrum colours shown in Figure 2.6. The three functions $\bar{x}$, $\bar{y}$ and $\bar{z}$ give the amount of three primaries (X), (Y) and (Z) respectively (i.e. the tristimulus values X, Y and Z), required to produce for the standard observer the colour of the spectrum at that wavelength (Judd and Wyszecki 1963; Wright 1964). By inserting the standard illuminant in the C.I.E. chromaticity diagram, it is possible

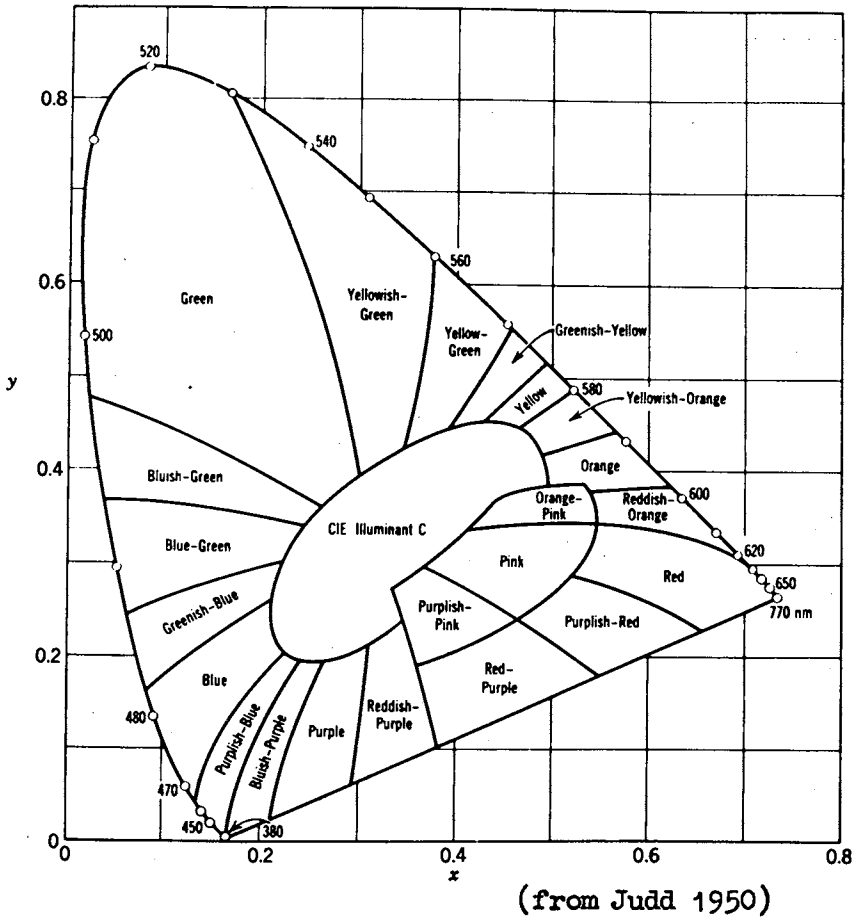
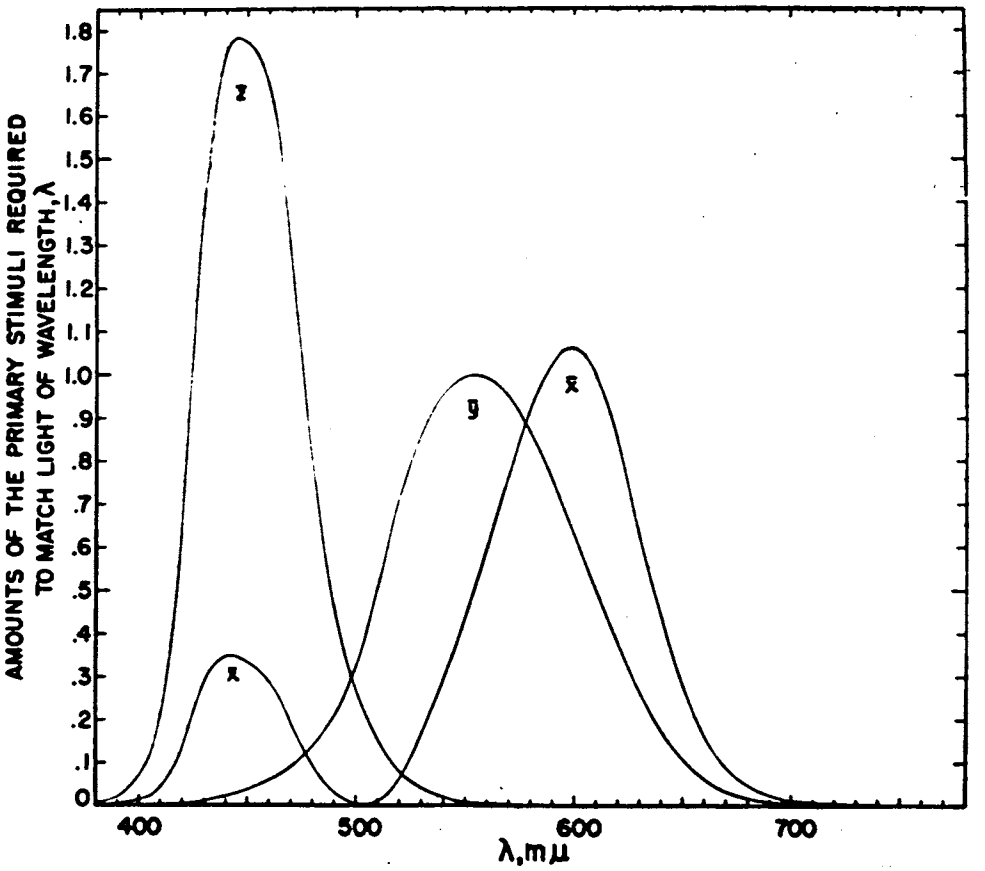


FIGURE 2.5

The (x,y)-chromaticity diagram of the C.I.E. system.



(from Judd 1950)

FIGURE 2.6

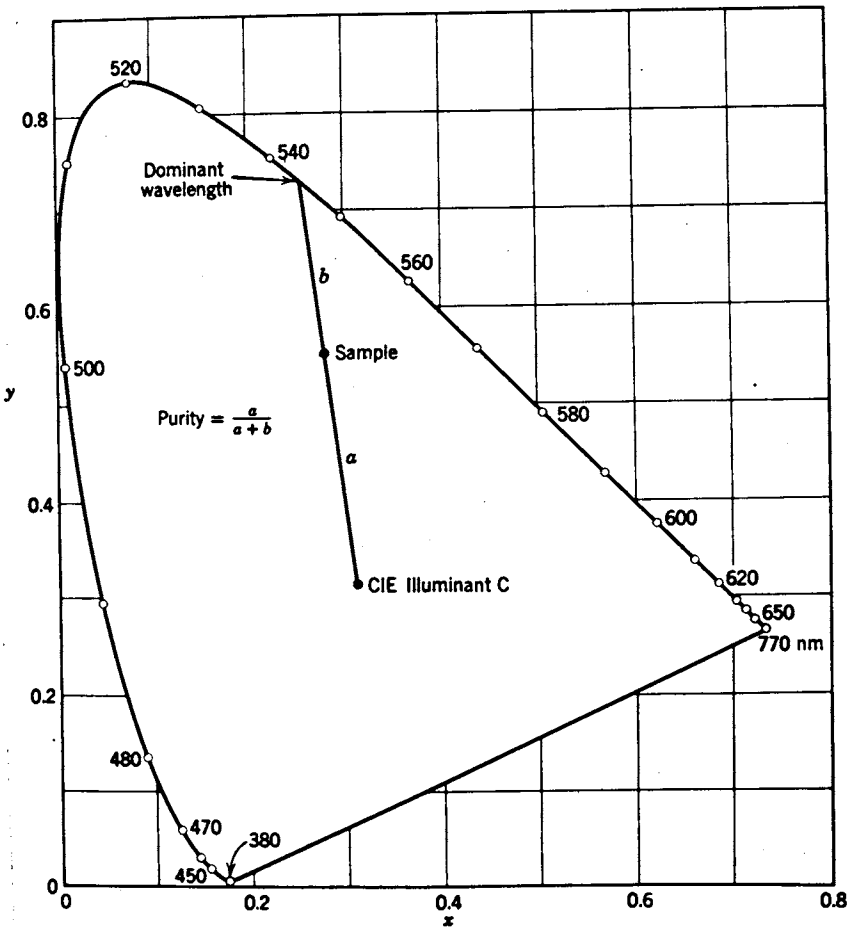
Tristimulus values of spectrum stimuli of unit irradiance according to the standard observer and co-ordinate system of the C.I.E. system.

to determine the visual dimensions of a particular colour. The hue of the colour is given in terms of the dominant wavelength located on the spectrum locus, while the saturation is measured in terms of purity, the distance from the illuminant to the sample divided by that from the illuminant to the spectrum locus (Figure 2.7). The lightness is given by the tristimulus Y value, perpendicular to the chromaticity plane.

The C.I.E. system has been extensively studied by many workers and has been shown to have certain disadvantages (Wright 1964). The most important is that equal differences in perceptibility between colours are represented by lines of unequal length in different parts of the colour space i.e. the C.I.E. chromaticity diagram is visually non-uniform. It is also very difficult to form a mental picture of a colour from its C.I.E. co-ordinates. These problems have been overcome to some extent by the use of colour spaces based on cylindrical or cartesian co-ordinates.

2.422 Munsell System

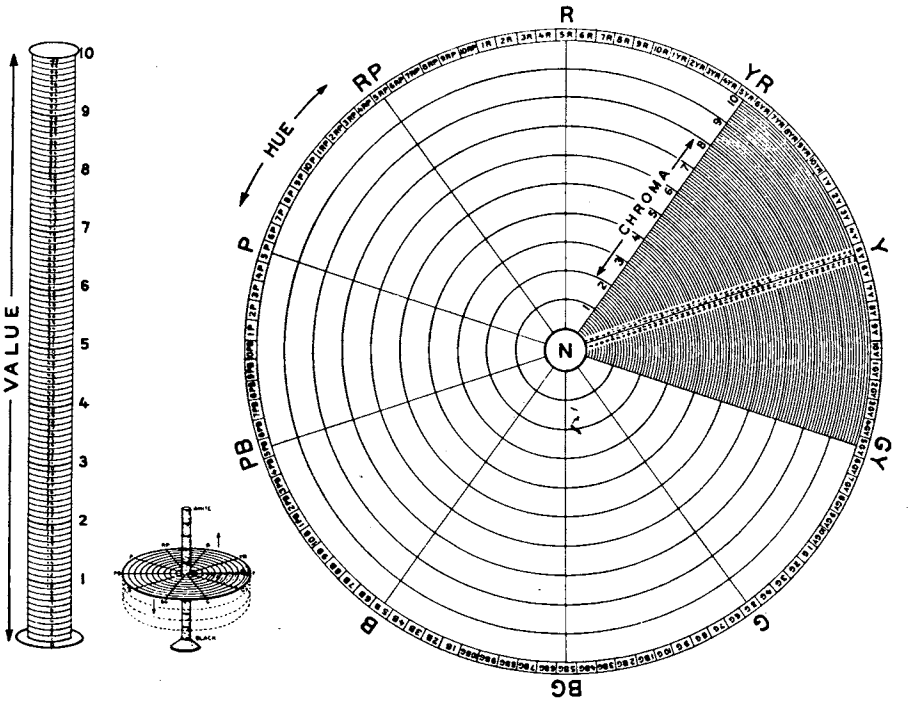
The Munsell system of colour notation was originally developed by A.H. Munsell (1858-1918) as an aid in teaching art (Nickerson 1940), but has since become of widespread use, particularly in the grading of agricultural products. In the Munsell notation, colour is expressed in units of visual difference of the three psychological attributes, hue, lightness and saturation. By this method results of colour measurement are expressed in terms of colour order rather than colour mixture (Nickerson 1946), and allow an interpretation of results directly in terms of the visual qualities known in the Munsell system as hue, value and chroma (Figure 2.8).



(from Billmeyer and Saltzman 1966)

FIGURE 2.7

The psychophysical quantities dominant wavelength and purity.



(from Nickerson 1946)

FIGURE 2.8

Munsell colour notation in scales of hue, value and chroma.

There are ten major Munsell hues (H), coded as R, Y, G, B, P (red, yellow, green, blue, purple) and the intermediates YR, GY, BG, PB and RP. Each is at the mid-point of a scale from 1 to 10, so that R is the same as 5R. Munsell value (V) is a lightness scale from 0 (Black) to 10 (White) through a series of intermediate greys. Munsell chroma (C) measures the difference of a colour from a grey of the same value. It is expressed on an arbitrary scale of intensity from 0 (white, grey or black) to as far as 18 for some hues. The Munsell notation for a colour is expressed as HV/C, where all three symbols can be decimal numbers. The Munsell system is different from the C.I.E. system in that perceptually equal differences in colour are represented by linear intervals on the Munsell colour solid. The Munsell system is used in the form of colour sheets or removable colour chips (Munsell Book of Colour 1965).

2.43 Measurement of Colour

Methods of colour measurement can be divided into subjective and objective techniques. The former depend on subjective evaluations or comparisons of colour by human observers, while the latter are based on instrumental determinations of colour or colour differences aimed to avoid the subjective character of visual methods. Various aspects of food colour and techniques of colour measurement have been reviewed by Mackinney and Chichester (1954), Quartermaster Food and Container Institute (1954), Little et al.(1958), Kramer and Twigg (1962), Mackinney and Little (1962) and Kefford (1963).

Visual methods of colour measurement include the use of colour dictionaries, or secondary standards such as painted rings, discs or

plastic models (Mackinney and Little 1962), subtractive colorimeters or tintometers, and disc colorimeters (Nickerson 1946). Methods of colour measurement and standardisation based on visual comparisons are subject to short-comings of human observers, such as the variability in the reaction of different observers, and in those of the one observer, at different times and under different viewing conditions, and the unreliability of colour memory. There have been many attempts, therefore, to use colour measurement methods which eliminate the human retina in favour of the photoelectric cell. A wide range of available instruments has been described by Livingston (1959) and Mackinney and Little (1962), and include reflectance spectrophotometers, photoelectric tristimulus colorimeters, and other instruments designed for highly specific purposes.

The historical development of photoelectric tristimulus colorimeters and colour-difference meters has been reviewed by Hunter (1942,1958) and Mackinney and Little (1962). Photoelectric instruments were originally designed in an attempt to measure directly the three C.I.E. tristimulus values X, Y and Z, without the laborious mathematics involved in converting readings from reflectance spectrophotometers. Three filter-photocell combinations were used having spectral sensitivity curves similar to the spectral distribution curves of the C.I.E. reference stimuli. Early instruments were limited, however, by the fact that available filter-photocell combinations did not reproduce perfectly the desired tristimulus functions. Many of these difficulties were overcome by using colour-difference meters in which photomultipliers, matched to different filters, simultaneously received light reflected by the specimen being measured.

One of the most successful instruments for measuring food colours is the Hunterlab Colour and Colour-Difference Meter. This instrument uses tristimulus amber, blue and green filters together with carefully chosen photocells and three separate metering circuits. Measurements according to the Hunter System locate colours on a colour space (Figure 2.9) related to but not identical with the C.I.E. colour space. The chromaticity plane is defined by dimensions 'a' and 'b' with the achromatic point at the origin. Positive 'a' values indicates redness, negative 'a' values greenness, positive 'b' values yellowness and negative 'b' values blueness. For any particular colour, the ratio a/b is a measure of the hue or dominant wavelength, while the saturation is approximated by the distance from the colour point to the origin i.e. $(a^2 + b^2)^{\frac{1}{2}}$. The lightness dimension perpendicular to the chromaticity plane passes through the origin and is calibrated in terms of diffuse reflectance (R_d) or lightness (L) in perceptibility units, depending on the circuitry in the instrument.

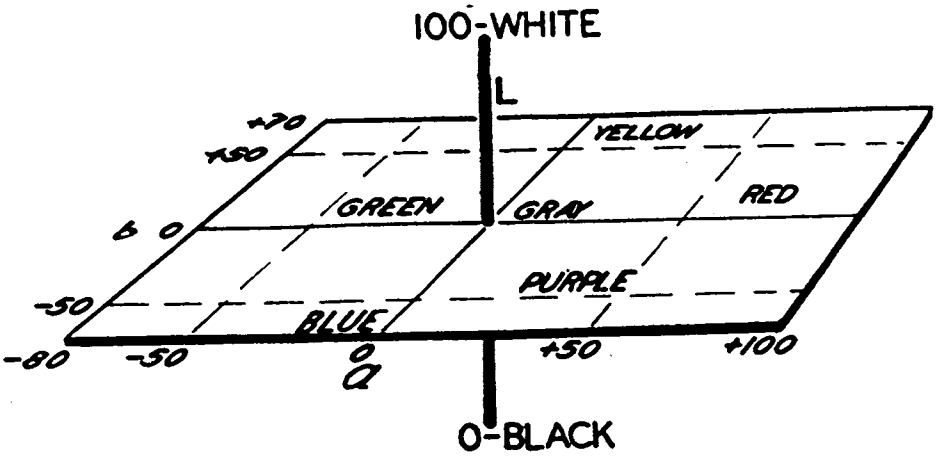
The relationship of L, a and b units to the X, Y and Z tristimulus values of the C.I.E. system are given by the following formulae (Esau 1958; Hunter 1958):

$$L = 100Y^{\frac{1}{2}}$$

$$a = 175Y(1.02X-Y)Y^{-\frac{1}{2}}$$

$$b = 70(Y-0.847Z)Y^{-\frac{1}{2}}$$

The N.B.S. unit of colour difference ΔE is given by the relation $\Delta E = (\Delta L^2 + \Delta a^2 + \Delta b^2)^{\frac{1}{2}}$, where ΔL, Δa and Δb are differences in values for L, a and b between a test and a control colour (Scofield 1943).



(from Hunter 1958)

FIGURE 2.9

The rectangular surface-colour solid with dimensions L, a and b.

Thus ΔE measures the distance between two points in the L, a, b rectangular solid, but does not specify the direction of the colour change.

2.44 Measurement of Colour and Colour-Difference of Chlorophyll-Containing Foods

2.441 Heat Processed Foods

The effects of heat processing and related techniques on the colour of chlorophyll-containing foods has been examined by a number of workers. Ross et al.(1959) showed that limits for colour variation in canned green beans, as specified by U.S. Department of Agriculture Grade Standards (1953), could be determined by simple statistical calculations based on objective colour data obtained with the Hunterlab Colour-Difference Meter. They gave an example of colour uniformity grading by these limits.

Gold and Weckel (1959) used a Hunterlab Colour-Difference Meter to measure objectively the colour of peas processed by H.T.S.T. methods. They found a significant correlation ($r = -0.922$) between percent chlorophyll conversion to pheophytin and the hue colour index $^{-a}/b$ measured by the Hunter instrument. A less significant correlation ($r = -0.388$) was found between the saturation index $(a^2 + b^2)^{\frac{1}{2}}$ and percent chlorophyll conversion. Subsequent work on H.T.S.T. processing of peas (Epstein 1959) has shown that various colour indices give reasonably good indications of visual colour before and after processing. Epstein measured by a Gardner Colour-Difference Meter the colour of H.T.S.T. processed peas and found that the loss of green colour during processing was best followed by plotting $^{-a}/b$ on a logarithmic scale against time on a linear scale to give a straight line relationship.

For spinach puree, however, a single colour function did not

correlate satisfactorily with visual colour determinations throughout the total storage time involved (Tan and Francis 1962; Gupte 1963; Clydesdale 1966). Initially, when the products were bright green, the function $\tan^{-1} a/b$ correlated with visual judgments, but when the puree became darker and more yellow due to the formation of pheophytins and pheophorbides, this function did not describe the actual appearance of the product. The indices 'L' and 'bL' did not correlate with visual judgments throughout the whole range of storage.

De Weese (1962) examined the effects of four processing methods on the quality of pureed snap beans, objective colour being measured by Hunterlab Colour and Colour-Difference Meter and the data converted to Munsell colour notations of hue, value and chroma. Munsell hue was highest (toward G from GY) in the lyophilised product, followed by the frozen, dehydrated and heat-processed products. The difference between each process was significant. Hue was influenced greatly by the chlorophyll b content, and the ratio C_a/C_b correlated well with hue. Munsell value had a negative correlation with total chlorophyll content, since the higher the total chlorophyll, the darker the colour. Munsell chroma was not significantly affected by the processes.

Pigment retention determinations by Sweeney and Martin (1958) were shown to be more closely correlated with panel scores and objective colour measurements for fresh and frozen broccoli than for cooked samples. Pigment methods which took into account the presence of pheophytins correlated more closely than methods for chlorophylls a and b only, when compared to visual or objective tests for colour. Cooked broccoli,

however, still showed good correlations between total chlorophyll and panel colour scores (Eheart and Gott 1965) and between total chlorophyll and Gardner Colour-Difference Meter 'a' values (Gilpin et al.1959).

2.442 Frozen Foods

Much information has been compiled by workers at the Western Regional Research Laboratory, U.S.D.A., California, on the effects of blanching and frozen storage on chlorophyll retention and colour, assessed visually by trained panels and objectively by colour-difference meters. Initial work (Lindquist et al.1950; Boggs and Talburt 1952) indicated that total chlorophyll and/or pheophytin was not closely associated with colour scores for fresh, frozen or dehydrofrozen peas, but significant correlations between pigment and colour data were reported in subsequent experiments. Dietrich et al.(1955) showed that changes in chlorophyll retention in stored frozen peas could be reasonably reflected by changes in Hunterlab 'a' values, and in particular, by the total colour-difference expression $\Delta E = (\Delta L^2 + \Delta a^2 + \Delta b^2)^{\frac{1}{2}}$. Boggs et al.(1960) reported that chlorophyll and Hunterlab 'a' values correlated reasonably well with industry panel scores, and that differences in 'a' values were good indices of colour deterioration.

Dietrich et al.(1957b) studied extensively some objective tests to measure adverse changes in frozen vegetables. They concluded, from results obtained with a Hunterlab instrument, that colour changes, measured objectively, differed markedly for peas and beans. The ratio $-a/b$, showing a change in hue from green to yellow, best represented changes in colour of green beans whereas the index $(a^2 + b^2)^{\frac{1}{2}}$ best

represented the colour changes in peas. Values of $(a^2 + b^2)^{\frac{1}{2}}$ for peas decreased with increasing storage time while $^{-a}/_{b}$ remained nearly unchanged in the early stages of storage and then decreased rapidly. Both indices for beans changed progressively with increasing storage, but the index $^{-a}/_{b}$ was considerably more consistent than changes in $(a^2 + b^2)^{\frac{1}{2}}$. These workers also found that the loss of chlorophyll appeared to be an excellent quality index since it closely paralleled adverse colour changes and other quality parameters. Quality changes in frozen spinach were subsequently examined (Dietrich et al.1960) and significant correlations were found between chlorophyll conversion, Hunterlab indices and panel evaluations of colour.

The establishment of an objective method of colour assessment focuses attention on its use in the grading of agricultural products, particularly for products in which colour is an obvious indication of quality. Objective methods have been used extensively for the grading of tomato products and many other agricultural commodities (Mackinney and Little 1962). Grading of materials such as fruits and vegetables containing chlorophylls is mostly done using food colour guides such as plates, discs, spatulas or rods of colours corresponding to the various grades of product available. Although this technique involves subjective human evaluation of colour, up to the present time it is still the most reliable method of assessing green colours associated with grading of foods containing chlorophylls.

3. PROCEDURE AND DISCUSSION OF RESULTS

3.1 PRELIMINARY EXPERIMENTS

Preliminary experiments aimed at establishing accurate and reliable methods for the preparation, purification and estimation of the chlorophyll pigments. It was mentioned previously that the most common method for the estimation of chlorophyll pigments involves spectrophotometry in organic solvents, and accordingly, instruments to be used for pigment estimation in the course of this work were checked for accuracy and reproducibility of wavelength and absorbance.

3.11 Calibration of Spectrophotometers

The instruments used most frequently in these investigations were a Unicam S.P. 600 manual spectrophotometer and a Unicam S.P. 800 double beam recording spectrophotometer. Wavelength accuracy was checked with standard filters, and corrections for the recording instrument applied by re-aligning the printed charts on the movable table (Expt.1). Occasionally, spectra were checked on a Cary 15 recording spectrophotometer. Absorbance accuracy was checked with acidified potassium dichromate, and also by a number of neutral glass filters obtained from the National Standards Laboratory, Sydney. The absorbance values of the filters measured by the two Unicam spectrophotometers (Table 4.01) show the manual instrument to be more accurate, particularly at high absorbance values. The largest error on this instrument was about 5% at an absorbance value in excess of 1.5. As absorbances of chlorophyll extracts were to be measured mostly in the range 0.1 - 0.8 units, the manual

instrument was considered satisfactory for use in the visible region (400-700nm).

3.12 Solvent Purification

Organic solvents except diethyl ether were of A.R. grade and were not purified further. Many workers have indicated the necessity for using pure solvents for pigment work, especially in the quantitative analysis of chlorophyll pigments and their derivatives using diethyl ether (Table 2.5). Since large quantities of diethyl ether were required for pigment analyses during the course of this work, it was considered uneconomical to purchase A.R. grade diethyl ether. Anaesthetic grade ether containing 0.002% w/v hydroquinone as a preservative, was purified by distillation and treatment with ferrous sulphate (Expt.2). Chlorophylls analysed in distilled ether gave results identical to the same samples in A.R. grade ether.

3.13 Chromatographic Column Packing

Several techniques of chromatographic column packing were attempted (Expt.3) in order to establish the most effective method dependent on the amount and type of pigment required. No single technique was found to be satisfactory for all adsorbents tested, and subsequently dry-packed and/or wet-packed columns of icing sugar, cellulose powder, or sugar/cellulose mixtures were used in various phases of the work. The most versatile adsorbent was found to be sugar-cellulose (icing sugar containing 10% powdered cellulose), since it gave separations equally as good as sugar but with the advantage of higher solvent flow rates. This adsorbent could be prepared by dry-packed or wet-packed methods, whereas

mixtures containing a higher proportion of cellulose (Jeffrey 1965) were suitable only for wet-packed columns.

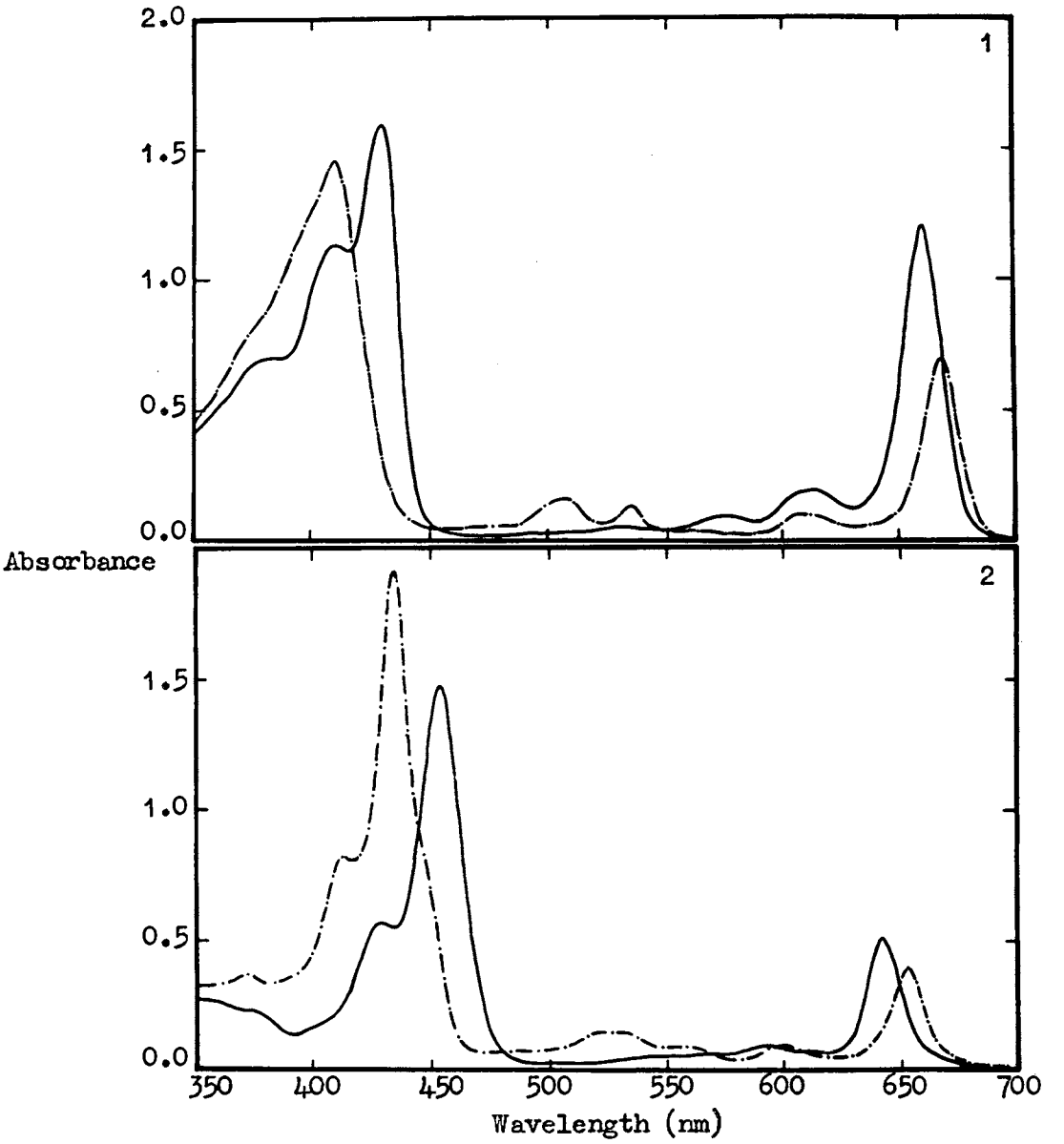
3.14 Purification of Chlorophyll Pigments

One of the most useful methods for the estimation of chlorophyll pigments and their derivatives involves spectrophotometry in organic solvents after extraction from the plant material. The alternative procedure involves chromatographic separation and isolation of each of the components in the pigment mixture and their subsequent estimation. The latter alternative was considered unsatisfactory for three reasons. Firstly, the chlorophyll pigments are labile reactive materials when removed from the plant source and could be expected to undergo some degradative changes during chromatography, particularly with thin layer chromatography, if atmospheric exposure was prolonged during development or elution. Secondly, no technique has been developed for the quantitative separation of a large number of chlorophyll derivatives in a single development. Estimation of breakdown products would then involve chromatography on 2 or more columns or T.L.C. plates using different adsorbents and/or developing solvents. Finally, because of the large number of samples to be analysed, both in Part I and Part II of this work, it was necessary to have a rapid method of pigment estimation which could be used repeatedly and carried out in a darkened room.

As previous work on H.T.S.T. processing of vegetable products had not reported the presence of the phytol-free derivatives chlorophyllides and pheophorbides (Gupte 1963), this aspect was included as a check on previous work. Consequently, the method described by White et al.(1963),

in which eight chlorophyll-type pigments can be estimated in the one solution, was considered to fulfil most of the requirements for the analysis of pigments. To check on the validity of the equations described in the above paper, it was necessary: (1) to prepare pure chlorophyll pigments and their derivatives, and compare wavelengths of maximum absorption and specific absorption coefficients with published data; and (2) to determine the accuracy of the estimation of several pigments in the one solution using the method of White et al.(1963).

Accordingly, chlorophyll pigments were extracted from fresh spinach leaves and chromatographed on icing sugar (Expt.4). Spectral data in distilled diethyl ether for purified chlorophyll a and pheophytin a, as well as chlorophyll b and pheophytin b (Graphs 1 and 2), are presented in Table 4.02. The results are summarised in Table 3.01, together with selected values reported in the literature (Table 2.5). The results showed good agreement to the previously reported figures of Zscheile and Comar (1941) which form the basis of several equations in the method of White et al.(1963). The values reported by Holt and Jacobs (1954) for the ethyl chlorophyllides a and b and their pheophorbide derivatives were also in satisfactory agreement with the values for the corresponding phytylated compounds, showing that the length of the side chain esterified to the C-7 propionic acid group did not produce any appreciable effect on the absorption curve of the pigments at the "red" absorption maximum. This assumption is used as the basis of the scheme proposed by White et al.(1963) (Figure 2.3). The close agreement of absorption maxima and specific absorption coefficients to published data indicate that distilled and purified anaesthetic ether could be used instead of A.R. grade ether.



Spectral curves of purified pigments in diethyl ether.

Graph 1 ——— chlorophyll a
-·-·- pheophytin a

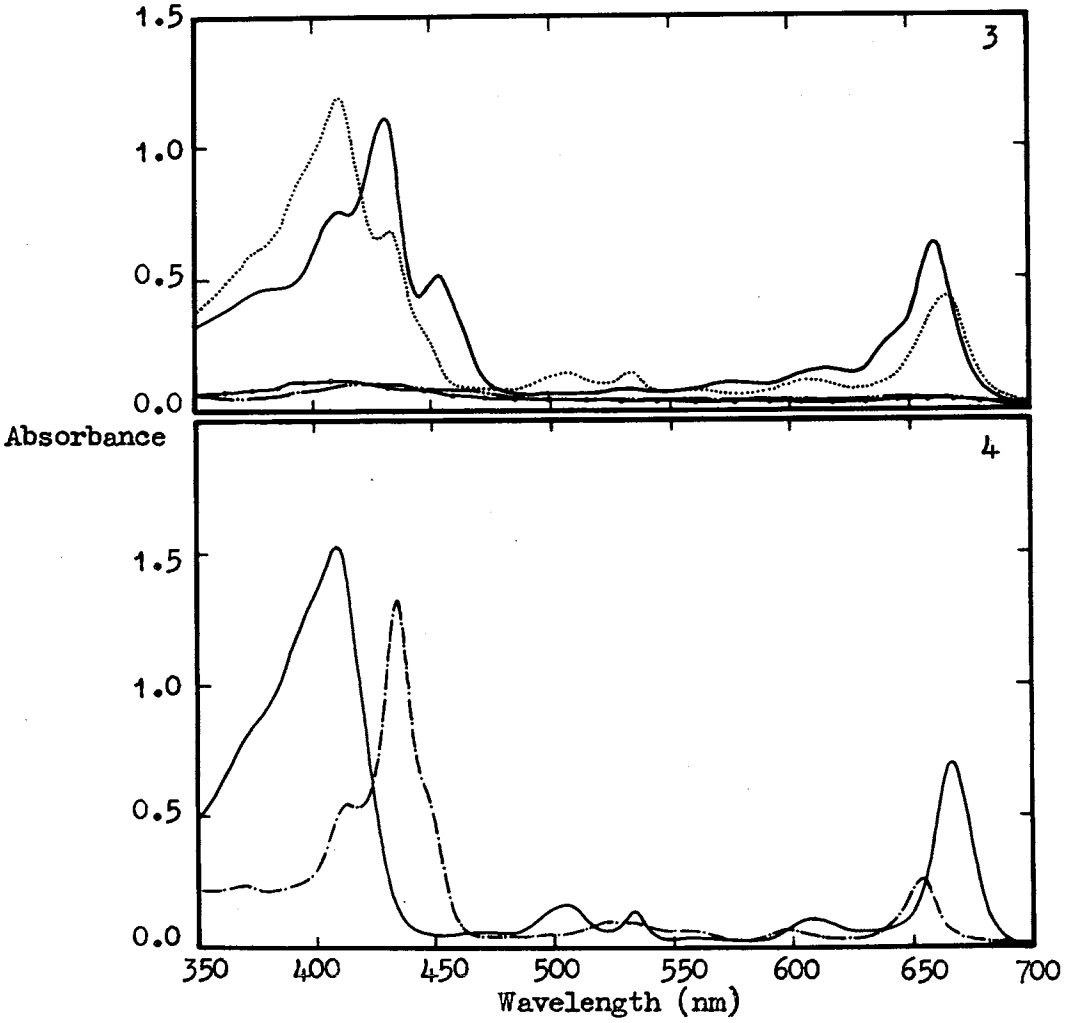
Graph 2 ——— chlorophyll b
-·-·- pheophytin b

Table 3.01 Absorption Measurements on Purified Chlorophyll Pigments in Diethyl Ether

Pigment	Red Absorption Maximum (nm)	Average Specific Absorption Coefficient (l/g.cm)	Literature Values		
			Absorption Maximum (nm)	Absorption Coefficient (l/g.cm)	Reference
Chlorophyll *a*	660	100.4	660	102.1	(1)
Ethyl Chlorophyllide *a*	-	-	660	100.1	(2)
Chlorophyll *b*	642.5	58.8	642.5	56.8	(1)
Ethyl Chlorophyllide *b*	-	-	640.5	58.0	(2)
Pheophytin *a*	667	58.95	666.5	59.0	(1)
Ethyl Pheophorbide *a*	-	-	666.7	57.9	(2)
Pheophytin *b*	653	37.7	653	37.0	(1)
Ethyl Pheophorbide *b*	-	-	653	37.0	(2)

(1) Zscheile and Comar (1941) (2) Holt and Jacobs (1954)

A crude chlorophyllide extract prepared from spinach by modifications of the method of Schanderl (1962) (Expt.5.1) was used without chromatographic separation of the individual pigments to examine the efficiency and reproducibility of extraction of phytol-free pigments by potassium hydroxide in the White et al.(1963) scheme. The spectra of the gross extract before and after acid conversion and alkali extraction are shown in Graph 3. Purified pheophorbides *a* and *b* obtained from purified pheophytins *a* and *b* respectively by acid hydrolysis (Expt.5.2, Table 4.03, Graph 4) showed almost identical absorption spectra to the parent pigments in agreement with the observations of Holt and Jacobs (1954). Pheophorbides *a* and *b* could be distinguished readily from the



Graph 3 Spectral curves of crude chlorophyllide solution in diethyl ether.

——— crude solution -·-· crude solution after alkali extraction

····· acidified solution -·-·- acidified solution after alkali extraction

Graph 4 Spectral curves of purified pigments in diethyl ether.

——— pheophorbide a -·-·- pheophorbide b

corresponding pheophytins a and b by underlayering ether solutions of the pigments with 22% hydrochloric acid. Pheophorbides were completely extracted by 22% acid, whereas the pheophytins remained in the ether phase. The hydrochloric acid numbers were characteristic for each pigment, being approximately 28-29 and 34-35 for pheophytins a and b respectively, and approximately 14-15 and 19-20 for pheophorbides a and b respectively.

Although it has been shown that acid hydrolysis sometimes yields small quantities of the pyropheophorbide derivatives, i.e. compounds in which the C-10 carbomethoxy group has been replaced by hydrogen, these were not removed by chromatography since they show identical absorption spectra to the parent pheophorbides (Pennington et al.1964) and would not interfere in tests or pigment determinations using the White et al.(1963) scheme.

3.15 Analysis of Pigments in Diethyl Ether

Solutions containing purified chlorophyll pigments (C_a, C_b, Py_a, Py_b, Po_a and Po_b) of known concentration were analysed by the method of White et al.(1963) (Expt.6, Table 4.05). Errors in the estimation of total pigment ranged from -6.4% to 5.9% which was considered satisfactory for the requirements of this investigation. Estimation of individual pigments, however, showed a greater variation in error as given in Table 3.02. All cases in which percentage errors exceeded $\pm$ 10% were in quantities of pigments representing less than 25% of the total pigment concentration. Errors in the estimation of C_b, Py_b and Po_b were greater than those found for the corresponding a pigments, as found by other workers, since absorbance measurements for the b compounds were taken

on a steep slope of the curve and not at the wavelength of maximum absorption of the gross extract. Small errors in locating the desired wavelength setting could thus produce significant absorbance errors.

Table 3.02 Errors in the Estimation of Chlorophyll Pigments in Diethyl Ether Using the Method of White et al.(1963)

Pigment	Range of Error (%)
Chlorophyll *a*	- 7.8 to 3.7
Chlorophyll *b*	- 2.6 to 20.4
Pheophytin *a*	- 5.6 to 6.0
Pheophytin *b*	-11.8 to 12.8
Pheophorbide *a*	-13.8 to - 3.4
Pheophorbide *b*	-10.6 to 10.8

Determination of the reproducibility of pigment extraction and estimation (Expt.6.2, Table 4.06), and efficiency of alkali extraction of phytol-free pigments from diethyl ether solutions (Table 4.07) showed the method of White et al.(1963) to fulfil the criteria described previously.

3.16 Analysis of Pigments in 80% Acetone

A method for the estimation of chlorophyll pigments and their derivatives in 80% acetone has been described by Vernon (1960). Pigment estimation in diethyl ether by the White et al.(1963) scheme involved extraction of the plant material with acetone and subsequent extraction of the pigments into ether from a solution initially containing about 80% acetone. The possibility of determining pigment concentrations by analysis in 80% acetone prior to ether extraction was therefore studied. Purified chlorophylls *a* and *b* and pheophytins *a* and *b* were dissolved in

80% acetone and their spectra recorded (Expt.7.1, Table 4.08). Absorption maxima were found to correspond closely to published figures of Vernon (1960) and others, and consequently this method was also used to estimate chlorophyll pigments in processed pea puree and frozen vegetables using the equations described (Expt.7.3).

The absorbance at 534nm of acidified pigment extracts in 80% acetone was found to differ by no more than 0.002 absorbance units from the value at the secondary absorption maximum in the pheophytin a spectrum (536nm), and consequently the method of Dietrich (1958) (Expt.7.2) was also adopted as a check on the estimation of % conversion of total chlorophyll to total pheophytin in gross extracts.

PART I. CHLOROPHYLLS AND COLOUR IN PROCESSED PEA PUREE

3.2 STORAGE STUDIES ON H.T.S.T. PROCESSED PEA PUREE

This section of the work was aimed at examining in detail the effects of pH elevation and H.T.S.T. processing, compared to conventional processing, on the degradation of chlorophyll pigments and its relationship to the visual colour of the processed product. In work on whole peas, Epstein (1959) examined the effects of processing and storage temperatures on changes in green colour, while the effects of processing temperature and product pH on pigment content and visual colour were determined for spinach puree (Tan and Francis 1962; Gupte 1963; Clydesdale 1966) for storage times up to 6 months at room temperature.

The following variables were used in the present work on pea puree:

Product pH: normal (pH6.95) and elevated (pH8.45)

Process temperatures: 240°F (conventional), 260°F, 280°F and 300°F

Process times: to give constant Fo value for each process temperature

Storage temperatures: 68°F, 37°F and -10°F

Storage times: up to 18 months.

Pea puree was used because of the availability and economic importance of the raw material, and to enable comparisons to be drawn with published data.

3.21 Preparation of Pea Puree

Fresh peas (Pisum sativum, variety Edgell Freezer) (150lb) grown in Tasmania, were hand picked and transported to the Kensington Laboratories (Expt.8.1). Maturometer readings taken shortly after picking (Table 4.09) showed that the peas were somewhat immature and suitable for freezing, although for canning a maturometer index in the range 230-270 is recommended for Grade 1 quality (Lynch and Mitchell 1953). The peas were blanched 1 minute in boiling water to inactivate peroxidase (Table 4.10), and pureed with or without the addition of magnesium carbonate (Expt.8.2) to a moisture content of 86.0-86.5%. Magnesium carbonate was used to elevate the pH since previous work (Gupte 1963) had showed that this material maintained attractive green colours and did not impair the flavour of the puree. Preliminary experiments with magnesium carbonate and other alkalysing agents verified these findings. It was noticed, however, that the pH-elevated puree was lighter in colour than the normal pH puree, due to the incorporation of the white powder as a suspension. This point is discussed more fully in Section 3.24.

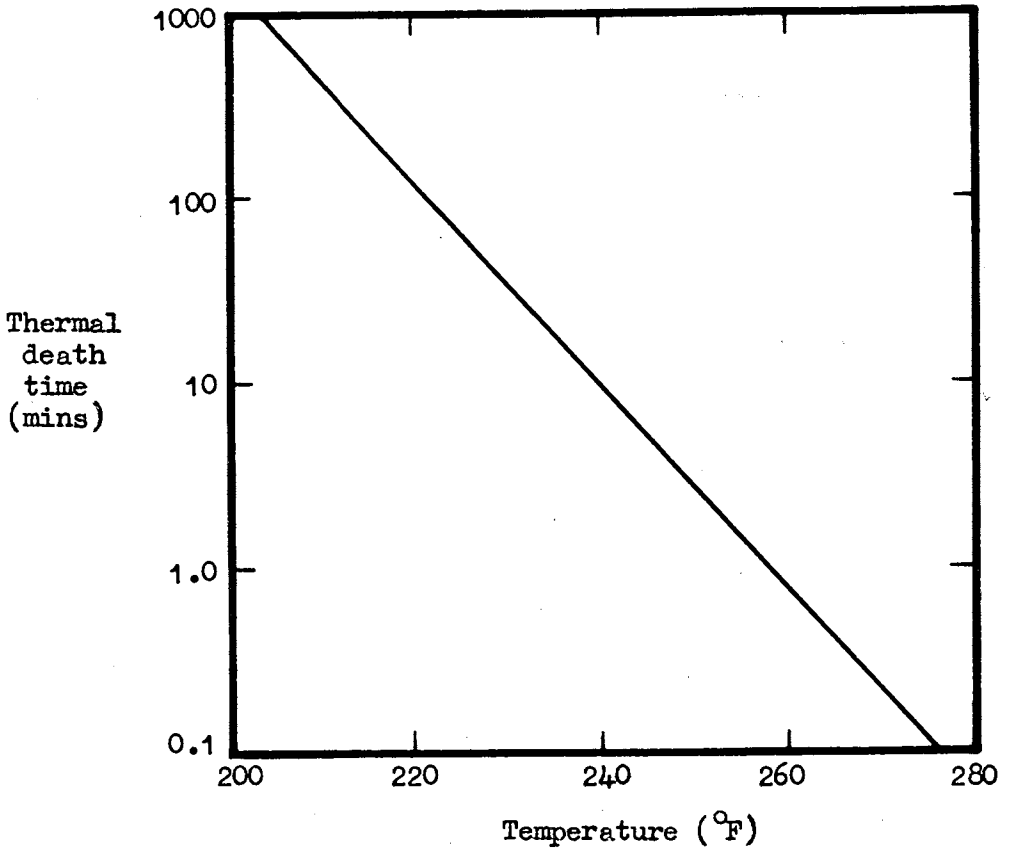
The puree was deaerated to remove excess air incorporated during blending and comminution, filled into Pyrex thermal death time (T.D.T.)

tubes by means of a syringe and sealed in an oxygen-coal gas flame. T.D.T. tubes were used because of their small size and hence rapid heat penetration.

3.22 Process Evaluation and Processing in T.D.T. Tubes

In order that puree processed at each of the process temperatures 240°F, 260°F, 280°F and 300°F be given an equivalent heat dose in terms of microbiological destruction, it was necessary to evaluate the rate of heat penetration into the slowest heating point of T.D.T. tubes containing pea puree at both pH values. This was carried out by inserting fine thermocouple wire into the centre of selected T.D.T. tubes and measuring the internal temperatures by direct-reading potentiometer (Expt.8.3). Average heat penetration data for each process temperature are given in Table 4.11. The incorporated magnesium carbonate had no apparent effect on the heat penetration rates, since no differences were found between normal or elevated pH samples.

The evaluation of process times required to give a constant lethal value at each process temperature was carried out by the graphical method of Bigelow et al.(1920). To simplify the calculations, three assumptions were made. These were:- (1) that the thermal death time for spores of Clostridium botulinum at temperatures greater than values given in the literature (N.C.A. 1954) could be obtained by extrapolation of the T.D.T. curve to higher temperatures (Graph 5); (2) that the elevation of the pH to 8.45 would not greatly affect the heat resistance of spores of Cl. botulinum; (3) that the actual lethal value of the processes was not critical, providing samples at each of the four temperatures were given identical lethal doses. These assumptions were made since it was desired



GRAPH 5

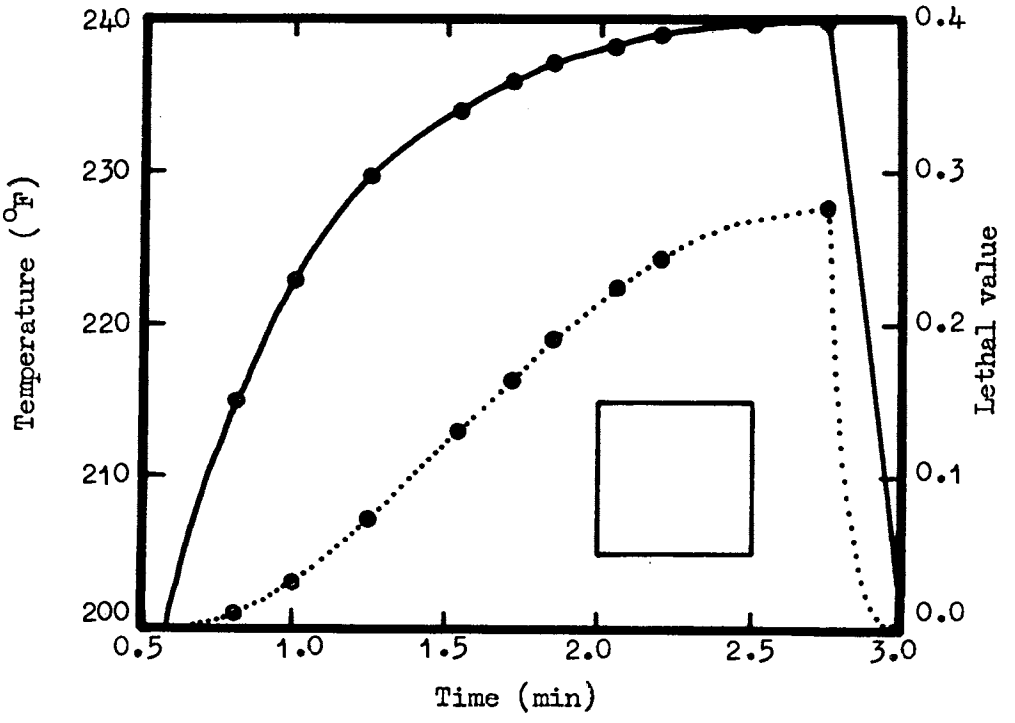
Extrapolated thermal death time curve for spores of Clostridium botulinum.
(data to 260°F from N.C.A. 1954).

only to observe the effects of heat processing, pH and storage on pigment and colour changes, and hence it was necessary to process both normal and elevated pH samples for identical times at each temperature. While it may be pointed out that some of these assumptions are not theoretically correct, the results of subsequent determinations showed that errors introduced by these assumptions were not significant.

The average heat penetration data for process temperatures 240°F, 260°F, 280°F and 300°F are shown in Graphs 6, 7, 8 and 9 respectively. Plotted on the same graphs are lethal value curves using lethal value data for various temperatures presented in Table 4.12. These lethal values represent the time in minutes at 250°F equivalent in sterilising value to one minute at other temperatures. Thus one minute at 240°F is equivalent (in terms of heat destruction of Cl.botulinum spores) to 0.1 minute at 250°F, hence the lethal value at 240°F is 0.1.

The calculation of process times at each of the process temperatures was based on F_o = 6.0, where F_o represents the area beneath a lethality curve of a process in terms of equivalent time in minutes at 250°F for the specific case where Z = 18°F. This value was chosen because it was comparable to commercial process values for non-acid products of similar consistency (N.C.A. 1955) and was higher than the process (F_o = 4.9) given to spinach puree processed under similar conditions (Tan and Francis 1962; Gupte 1963) which was identical to commercial processes in baby food jars. Since peas are slightly less acid than spinach, this assumption appears reasonable.

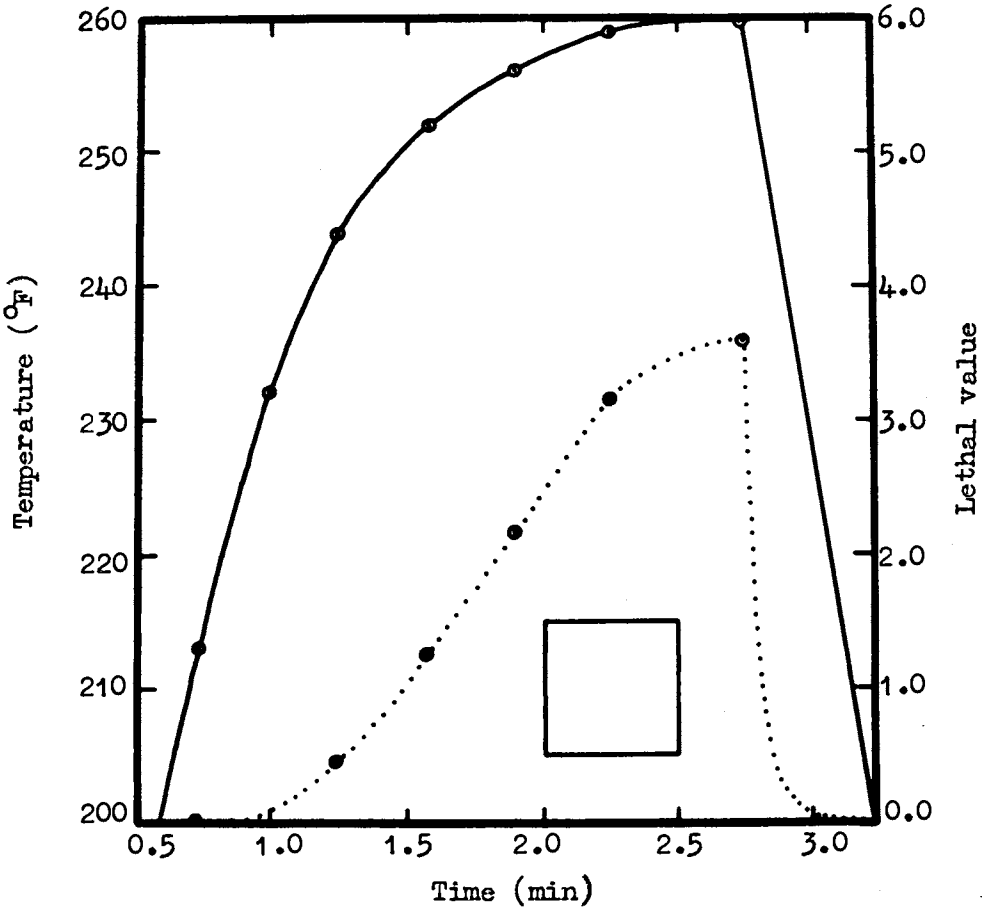
The process times required to give F_o = 6.0 at each temperature



GRAPH 6

Heat penetration (———) and lethal value (.....) curves for pea puree processed in T.D.T. tubes in an oil bath at 240°F.

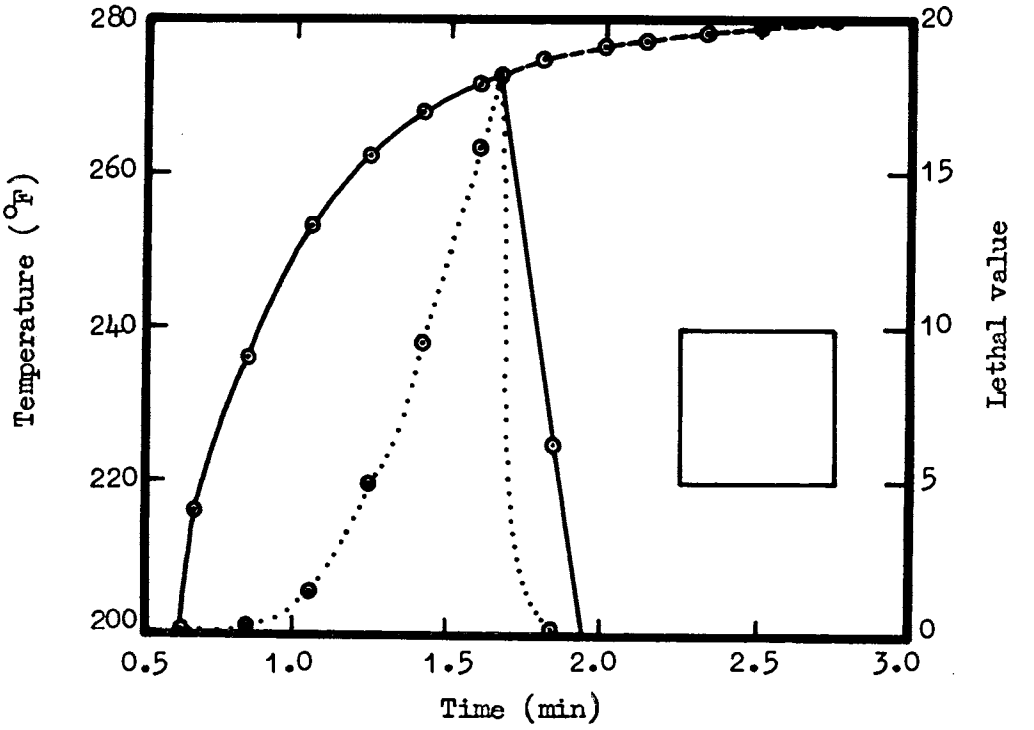
The enclosed area represents F_o = 0.05.



GRAPH 7

Heat penetration (———) and lethal value (.....) curves for pea puree processed in T.D.T. tubes in an oil bath at 260°F.

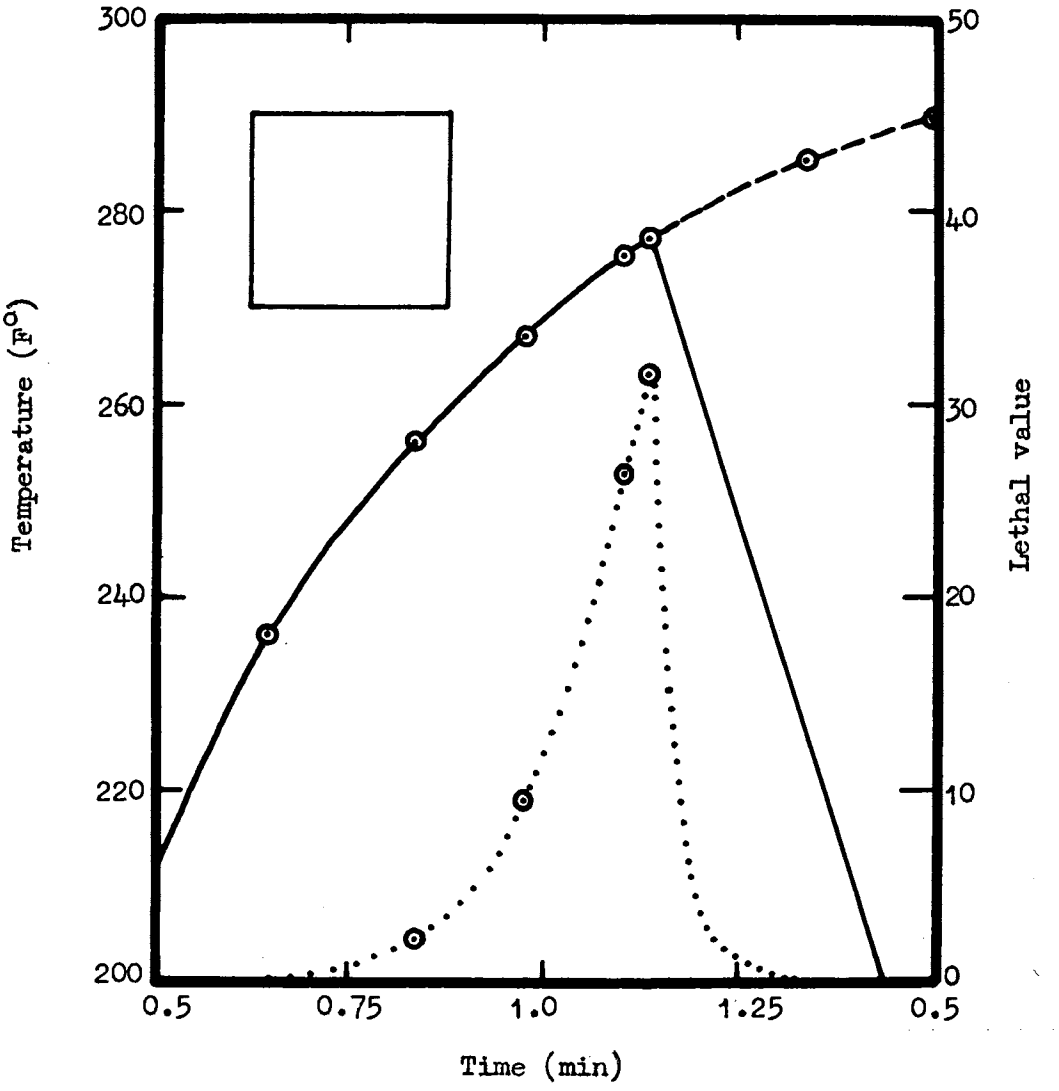
The enclosed area represents F_o = 0.5.



GRAPH 8

Heat penetration (———) and lethal value (.....) curves for pea puree processed in T.D.T. tubes in an oil bath at 280°F.

The enclosed area represents F_o = 2.5.



GRAPH 9

Heat penetration (——) and lethal value (.....) curves for pea puree processed in T.D.T. tubes in an oil bath at 300°F.

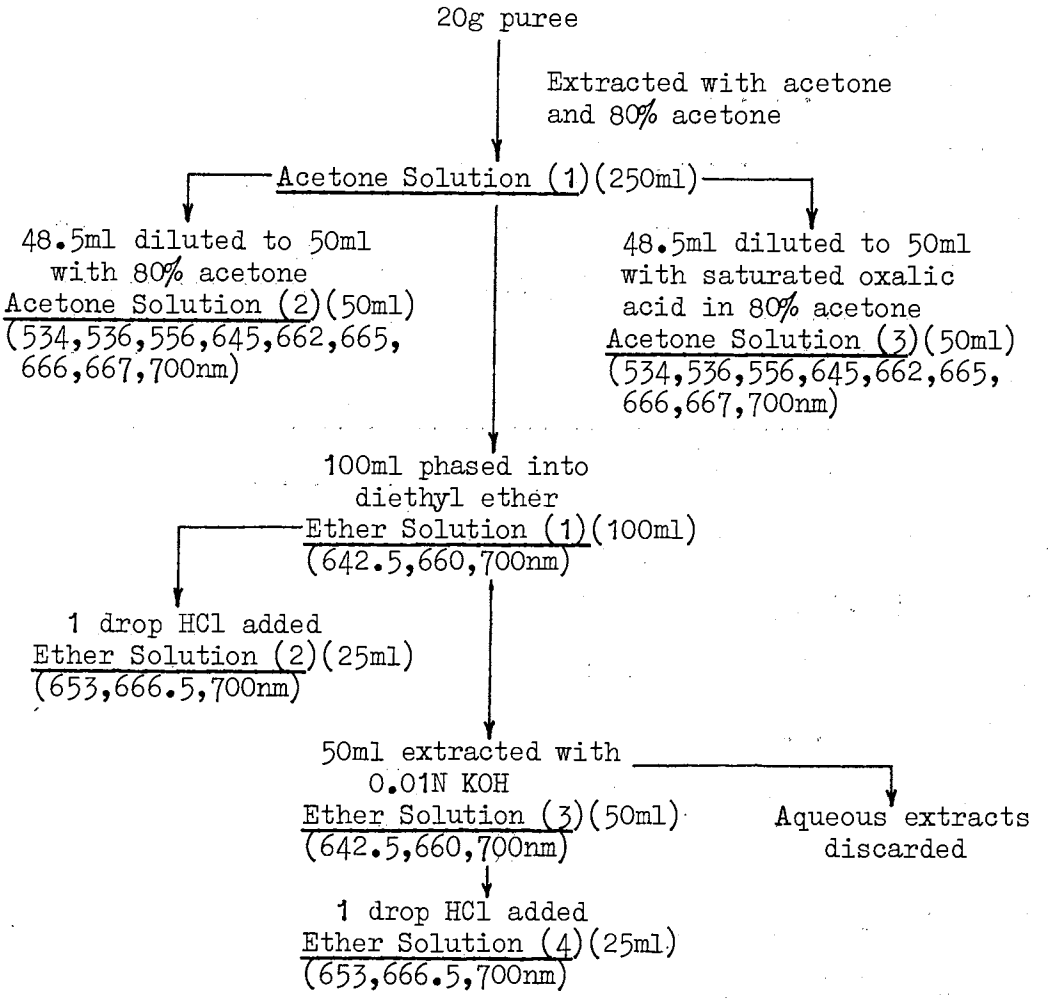
The enclosed area represents F_o = 2.5.

were in agreement with similar work on spinach puree. The much shorter process times required for high temperature processing is a consequence of the logarithmic thermal death time for bacterial spores (Graph 5), and outlines the advantages to be gained by H.T.S.T. processing compared to conventional processing (e.g. 240°F). T.D.T. tubes containing normal and elevated pH puree were subsequently processed and placed on storage at 68°F, 37°F and -10°F (Table 4.13) to be analysed for pigments, colour and pH.

3.23 Pigment Changes in Processed and Stored Pea Puree

Following H.T.S.T. processing, samples of pea puree were placed in storage and subsequently analysed at time intervals up to 18 months. Pigments were extracted in the dark with acetone (Expt.9.1) and pigment contents determined in 80% acetone (Expt.9.2) and diether ether (Expt. 9.3) according to the scheme presented in Figure 3.1. The bracketed figures indicate wavelengths (nm) at which absorbance values were determined. The extensive results of pigment determinations on normal and elevated pH pea puree are presented in Tables 4.14 to 4.25 inclusive (Expt.10). These results are more easily examined if the effects of process temperature, storage temperatore and product pH are discussed separately. The presence of phytol-free derivatives (chlorophyllides or pheophorbides) could not be established in any sample, in agreement with the results of Gupte (1963).

Figure 3.1 Scheme for the Estimation of Chlorophyll Pigments in Pea Puree



3.231 Effects of Process Temperature and pH

Results of the effects of process temperature on the rate and extent of chlorophyll destruction in processed puree were in agreement with published work, in that higher process temperatures gave less pigment destruction in terms of conversion of chlorophylls (total or individual) to pheophytins (total or individual) at both normal and elevated pH values. These results are summarised in Table 3.03.

Table 3.03 Effect of Process Temperature on Total Chlorophyll and Percent Chlorophyll Conversion in Normal and Elevated pH Pea Puree

Puree pH	Process Temperature (°F)	Total Chlorophyll		Chlorophyll a		Chlorophyll b	
		Pigment Content (µg/g)	Percent Conversion	Pigment Content (µg/g)	Percent Conversion	Pigment Content (µg/g)	Percent Conversion
6.95	Unprocessed	86.0	4.0	60.8	5.6	25.2	0.0
"	240	15.5	83.2	5.5	91.7	10.0	60.8
"	260	61.8	30.1	38.3	39.2	23.5	7.8
"	280	78.3	14.6	55.6	10.5	22.7	9.6
"	300	76.0	16.3	51.9	19.3	24.1	9.1
8.45	Unprocessed	77.2	4.0	54.6	5.5	22.6	0.0
"	240	51.4	34.9	35.0	39.5	16.4	25.5
"	260	67.3	15.8	48.0	17.5	19.3	11.1
"	280	76.4	8.1	53.6	10.1	22.8	3.0
"	300	73.0	10.7	51.8	11.9	21.2	7.4

It is seen that for either normal or elevated pH samples, processing at 240°F, representing a conventional operation, resulted in more than twice the (total) chlorophyll conversion found in samples processed at 260°F for periods less than one quarter the total process time. Increasing the process temperature to 280°For 300°F effected another 50% reduction in chlorophyll conversion, but no significant difference was evident between the two highest process temperatures. The slightly higher pigment content in puree processed at 280°F, compared to 300°F, was probably due to difficulty in maintaining specified process times at this temperature, or to a small error in the calculation of the process time. The difference was minor, however, and emphasised that process temperatures above 280°F would give only marginal benefits.

The different rates of conversion of chlorophylls a and b to their respective magnesium-free derivatives were also in agreement with data reported in the literature for other food products containing chlorophylls (Sweeney and Martin 1958,1961; Tan and Francis 1962; Gupte et al. 1964; Clydesdale 1966; Eheart 1967). The rates of conversion were not of the same order, however, as found in in vitro systems containing pure pigments (Joslyn and Mackinney 1938; Schanderl et al.1962) and it would appear that the reactivity of chlorophyll a in vivo is markedly reduced when associated with proteins, lipids and other materials. In all cases, however, it was found that chlorophyll a degraded at a faster rate than chlorophyll b.

It was also observed that chlorophyll conversion was substantially reduced at each process temperature by elevation of the pH from 6.95 to 8.45 before processing. The effect of increasing the pH on the retention of green chlorophyll colour in processed foods has been known for some time, and several patents have been issued for processes in which alkalysing steps were included (Blair 1940a,b; Malecki 1957,1964). These processes did not find general acceptance, however, because of claims that storage stability after processing was severely limited.

The influence of process temperature and pH on pigment composition is also shown in Table 3.04 for various pigment ratios. In all cases, values for samples processed at 240°F at either pH value were considerably less than the values for higher process temperatures, while the elevated pH samples showed greater chlorophyll retention than samples at normal pH.

Table 3.04 Effect of Process Temperature on Pigment Ratios in Normal and Elevated pH Pea Puree

Puree pH	Process Temperature (°F)	Pigment Ratios			
		C_a/C_b	C_a/Py_a	C_b/Py_b	Py_a/Py_b
6.95	Unprocessed	2.41	16.89	-	-
"	240	0.55	0.09	0.65	3.94
"	260	1.63	1.55	11.75	12.35
"	280	2.45	5.05	9.46	4.58
"	300	2.15	4.19	10.04	5.17
8.45	Unprocessed	2.42	17.06	-	-
"	240	2.13	1.60	2.93	3.91
"	260	2.49	4.71	8.04	4.25
"	280	2.39	8.93	12.57	8.57
"	300	2.44	7.40	12.47	4.12

3.232 Effect of Storage Time and Temperature

The effects of storage time and storage temperature on the content of chlorophyll pigments in processed normal pH pea puree are summarised in Table 3.05 in which pigment data is expressed as percent conversion of total chlorophyll. It is seen that complete (100%) conversion of chlorophylls to pheophytins occurred with all samples after 18 months storage at 68°F. All samples showed more than 90% conversion after 6 months storage with 100% conversion at the lower process temperatures (240°F and 260°F). Even after 2 months storage, all samples showed more than 85% conversion despite the much greater initial retention of chlorophyll in samples processed at 280°F and 300°F. All samples stored at 37°F showed at least 85% conversion after 12 months storage, and after 18 months, only the commercially processed (240°F) sample showed com-

plete conversion. This observation was in agreement with the expected effect of temperature on chemical reactions. Samples stored for 18 months at freezing temperatures (-10°F) showed no significant changes in pigment content or pigment distribution.

Table 3.05 Percent Chlorophyll Conversion During Storage of Normal pH Pea Puree

Storage Temperature (°F)	Storage Time (months)	Percent Chlorophyll Conversion			
		Process Temperature (°F)			
		240	260	280	300
Unprocessed		4.0	4.0	4.0	4.0
Processed		83.2	30.1	14.0	16.3
68	2	198.6	91.3	89.2	86.9
"	3	100	94.1	95.3	90.1
"	6	100	100	98.2	90.8
"	12	100	100	95.1	100
"	18	100	100	100	100
37	3	90.1	73.3	54.6	57.2
"	6	96.2	85.6	71.8	70.2
"	12	100	90.3	84.9	87.1
"	18	100	98.1	91.8	93.4
-10	6	88.7	28.7	18.3	17.2
"	12	86.6	31.4	15.9	15.2
"	18	85.3	32.8	16.8	17.8

The effect of storage temperature on the rate of chlorophyll conversion in processed puree at normal pH is more readily seen in Table 3.06, in which the percent chlorophyll conversion due to processing and storage are tabulated separately. It is seen that for samples processed at 240°F, 83.2% conversion occurred during preparation and processing and a further 16.8% during storage at 68°F and 37°F. However, the 16.8%

conversion during storage took place in 3 months at 68°F, of which 91.7% (i.e. 15.4% out of 16.8%) occurred during the first 2 months. At 37°F, 12 months storage was required before 100% conversion took place. Less than half of the 16.8% conversion (i.e. 6.9%) that occurred during 18 months storage took place during the first 3 months.

Table 3.06 Effect of Processing and Storage on Percent Chlorophyll Conversion in Normal pH Pea Puree

Storage Temperature (°F)	Chlorophyll Conversion Due to	Percent Chlorophyll Conversion			
		Process Temperature (°F)			
		240	260	280	300
-	Preparation and Processing	83.2	30.1	14.6	16.3
68	2 months storage	15.4	61.2	74.6	70.6
"	3 " "	16.8	64.0	80.7	73.8
"	6 " "	16.8	69.9	83.6	74.5
"	12 " "	16.8	69.9	80.5	83.7
"	18 " "	16.8	69.9	85.4	82.1
37	3 " "	6.9	43.2	40.0	40.9
"	6 " "	13.0	55.5	57.2	53.9
"	12 " "	16.8	60.2	70.3	70.8
"	18 " "	16.8	68.0	77.2	77.1

For higher process temperatures, the situation was similar in that during storage at 68°F, the largest proportion of the conversion which occurred during the whole storage period (i.e. 18 months), took place in the first 2 months. As the process temperature was increased to 260°F, 280°F and 300°F, however, this proportion decreased, showing that higher process temperatures resulted in a greater initial retention of chlorophyll during the early storage periods. It is of interest to note, however, that while high process temperatures resulted in a much

greater retention of chlorophyll, the residual chlorophyll remaining was degraded at approximately the same rate during storage, i.e. the rate was independent of the residual chlorophyll concentration, and chlorophyll loss during storage followed first-order kinetics. Thus for normal pH puree stored at 68°F, of the chlorophyll degraded during storage for 18 months at 240°F, 260°F, 280°F and 300°F, the proportion lost during the first 2 months storage was 91.7%, 87.6%, 87.4% and 86.0% respectively, although five times as much total chlorophyll was present in the 300°F sample (76.0 µg/g) than in the 240°F sample (15.5 µg/g) at the start of the storage period. For storage at 37°F, the proportions lost during the first 3 months compared to the total storage loss over 18 months were 41.1%, 63.5%, 51.8% and 53.0%, with increasing process temperatures.

At higher process temperatures more chlorophyll was initially retained, consequently there was more chlorophyll present to be destroyed during storage. This would explain the observations of other workers that the colour of H.T.S.T. processed samples was less stable than samples processed by conventional methods. Since more chlorophylls were retained after H.T.S.T. processing, their conversion during storage resulted in more noticeable colour changes than in conventionally processed samples in which most of the pigment was converted during processing.

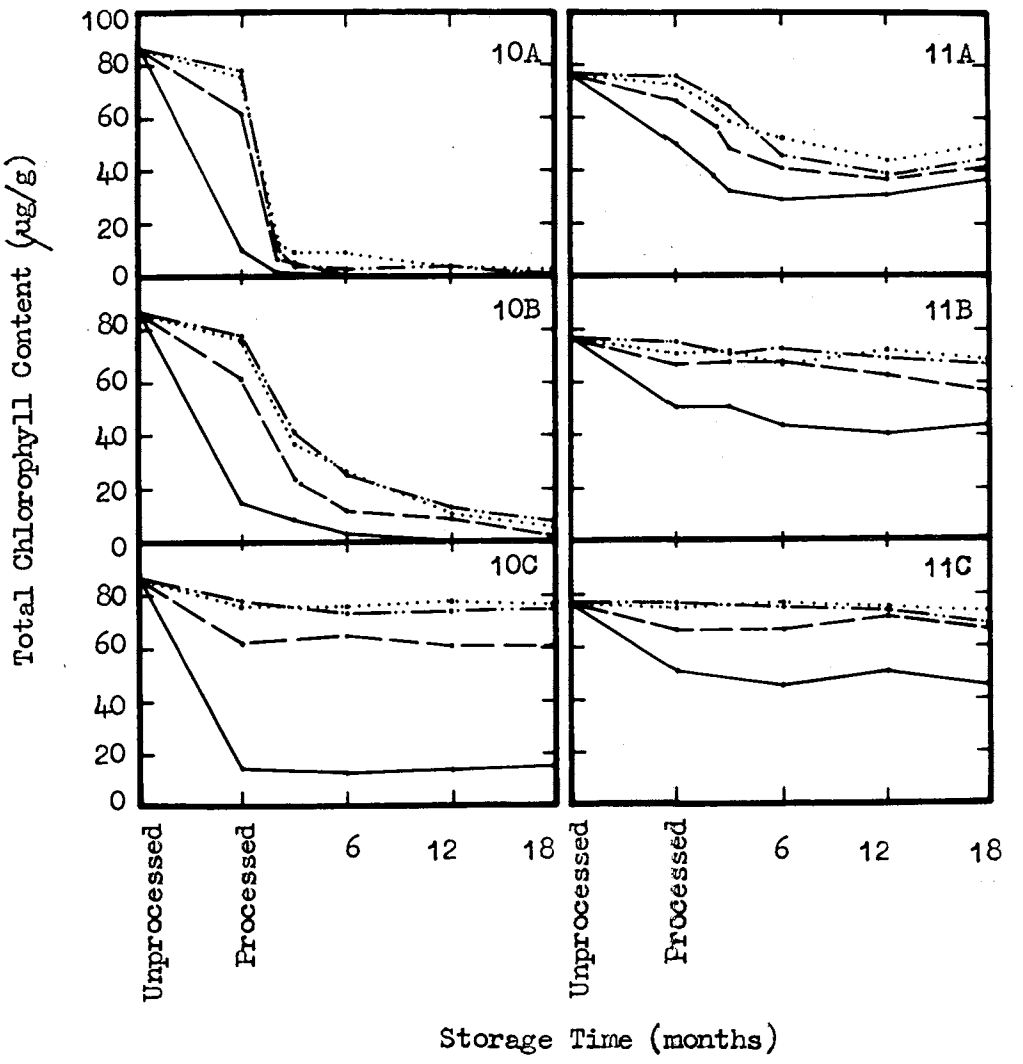
The effects of storage temperature and storage time on the pigments of processed elevated pH pea puree are summarised in Table 3.07. It is seen that storage temperature exerted a similar effect on pigment degradation in elevated pH puree as in normal pH puree, but the effects

were of a smaller magnitude. Again, higher process temperatures and lower storage temperatures gave less conversion of chlorophyll to pheophytin. At low storage temperatures (37°F and -10°F) and high process temperatures (260°F, 280°F and 300°F), storage times up to 18 months produced only very small increases in percent conversion of chlorophylls.

Table 3.07 Percent Chlorophyll Conversion During Storage of Elevated pH Pea Puree

Storage Temperature (°F)	Storage Time (months)	Percent Chlorophyll Conversion			
		Process Temperature (°F)			
		240	260	280	300
Unprocessed		4.0	4.0	4.0	4.0
Processed		34.9	15.8	8.1	10.7
68	2	51.7	30.1	20.5	10.3
"	3	58.1	35.5	21.5	24.8
"	6	65.6	49.2	40.1	35.1
"	12	58.8	53.3	48.9	45.1
"	18	52.3	47.4	41.8	39.7
37	3	35.7	18.3	12.8	10.3
"	6	45.9	15.4	10.8	14.3
"	12	48.4	18.1	15.7	10.2
"	18	41.3	27.7	18.1	14.2
-10	6	42.3	16.5	8.7	10.9
"	12	38.1	10.4	10.4	7.1
"	18	44.6	14.3	12.7	13.2

The changes in total chlorophyll content of normal and elevated pH puree during processing and 18 months storage are shown in Graphs 10 and 11, from which the effects of process temperature, storage temperature, storage time and product pH can be analysed separately.



Effect of processing and storage on total chlorophyll content of pea puree.

GRAPH 10 Normal pH

GRAPH 11 Elevated pH

A	Storage Temp.	68°F	———	Process Temp.	240°F
B	" "	37°F	– – –	" "	260°F
C	" "	-10 F	–·· –	" "	280°F
			·····	" "	300°F

3.233 Unusual Pigment Results with Elevated pH Pea Puree

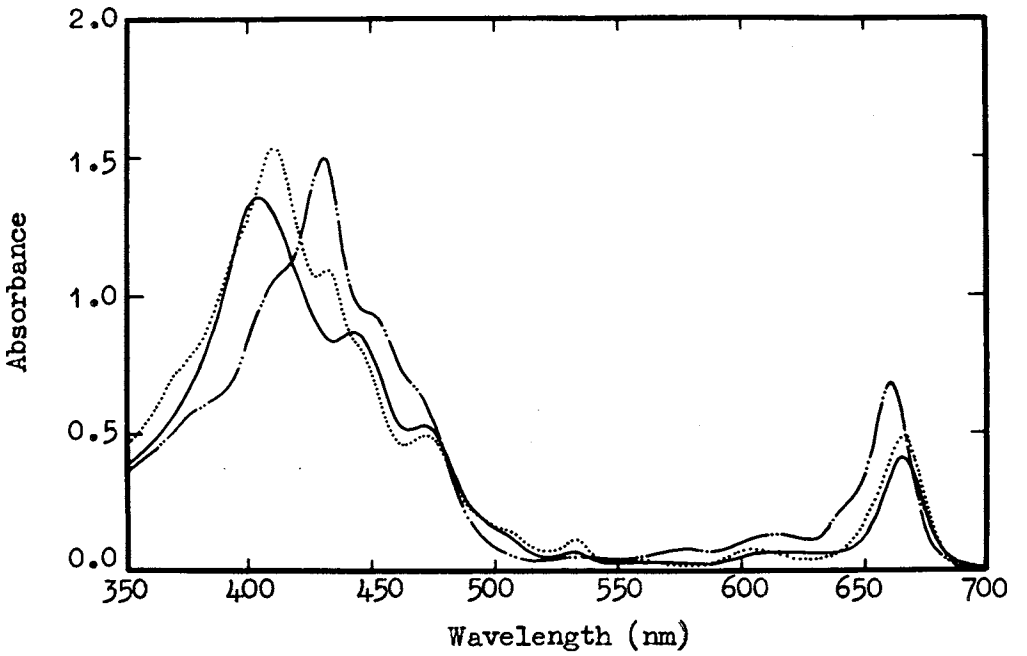
Examination of pigment data for elevated pH pea puree stored for up to 18 months at 68°F (Tables 4.14 to 4.17) and at 37°F (Tables 4.18 to 4.21) showed several unusual results. Samples analysed after 12 and 18 months storage at 68°F for all process temperatures showed increased concentrations of chlorophyll a and lower values for percent conversion of total chlorophyll to pheophytin and chlorophyll a conversion to pheophytin a compared to pigment results after 6 months storage. Samples analysed after 12 and 18 months storage at 37°F also showed high concentrations of chlorophyll b, and low values for percent conversion of total chlorophyll and chlorophyll a compared to samples analysed after shorter storage periods. These trends are shown by examining the pigment ratios Total a/Total b, C_a/Py_a, C_b/Py_b and Py_a/Py_b for each process temperature and storage time for samples stored at 68°F. The results are presented in Table 3.08. The obvious changes are the smaller ratio of T_a/T_b, the increases in the ratios C_a/C_b and C_a/Py_a, and the decreases in the ratios C_b/Py_b and Py_a/Py_b after 12 and 18 months storage. Since the total pigment concentration did not change significantly, the changes in the pigment ratios can be attributed to an increase in the concentrations of chlorophyll b and/or pheophytin b, or a decrease in the concentration of chlorophyll a and/or pheophytin a, or a combination of both factors.

Table 3.08 Pigment Ratios in Extracts from Elevated pH Pea Puree Processed by H.T.S.T. Methods and Stored 18 months at 68°F

Process Temperature (°F)	Pigment Ratio	Unprocessed	Storage Time (Months) 0	2	3	6	12	18
240	T_a/T_b*	2.56	2.59	2.39	2.49	2.36	1.63	1.29
"	C_a/C_b	2.42	2.13	1.62	1.77	1.07	1.23	1.98
"	C_a/Py_a	17.06	1.60	0.74	0.60	0.34	0.58	1.06
"	C_b/Py_b	-	2.93	1.67	1.11	1.27	0.95	0.76
"	Py_a/Py_b	-	3.91	3.67	3.28	3.99	2.01	1.10
260	T_a/T_b*	2.56	2.69	2.50	2.54	2.29	1.68	1.42
"	C_a/C_b	2.42	2.49	1.80	1.97	1.33	1.12	1.56
"	C_a/Py_a	17.06	4.71	1.70	1.48	0.72	0.65	1.21
"	C_b/Py_b	-	8.04	6.93	3.32	2.52	1.44	0.99
"	Py_a/Py_b	-	4.25	7.37	4.42	4.70	2.48	1.28
280	T_a/T_b*	2.56	2.53	2.49	2.51	2.48	1.88	1.37
"	C_a/C_b	2.42	2.35	2.23	3.08	1.86	0.82	1.41
"	C_a/Py_a	17.06	8.93	3.34	5.34	1.21	0.54	1.43
"	C_b/Py_b	-	32.57	6.03	1.83	2.67	4.27	1.34
"	Py_a/Py_b	-	8.57	4.03	1.05	4.11	6.43	1.32
300	T_a/T_b*	2.56	2.57	2.68	2.39	2.51	1.78	1.50
"	C_a/C_b	2.42	2.44	2.43	2.51	1.88	1.00	1.21
"	C_a/Py_a	17.06	7.40	3.73	3.21	1.45	0.75	1.23
"	C_b/Py_b	-	12.47	5.94	2.67	3.74	3.21	2.13
"	Py_a/Py_b	-	4.12	3.88	2.08	4.84	4.29	2.10

* Ratio of Total a to Total b pigments

Subsequent examination of spectra of gross extracts from samples of normal and elevated pH pea puree stored for 18 months at 68°F and -10°F (Graph 12) showed the errors to be due to the use of incorrect absorbance values using the equations of Dietrich (1958), Vernon (1960) and White et al.(1963). Comparison of the spectra of gross extracts from normal and elevated pH pea puree stored 18 months at 68°F showed that the "red"



GRAPH 12

Spectral curves of gross pigment extracts from processed and stored pea puree.

——— elevated pH puree stored 18 months at 68°F
- - - - elevated pH " " " " " -10°F
..... normal pH " " " " " 68°F

absorption maximum had shifted from 667nm to about 664nm in diethyl ether, and the intensity of absorption had decreased. There was also a substantial decrease in absorption in the region near 534nm, and a change in the absorption pattern near 630-640nm. Absorption in the "blue" region showed a very unusual maximum at about 402nm, much lower than the usual pheophytin a peak at 408-410nm, while the pheophytin b peak at 434nm was considerably reduced.

These observations indicated that a considerable alteration of the pigment pattern in elevated pH pea puree had occurred, presumably due to the alkaline pH of the puree. The greater changes apparent in samples stored at 68°F compared with samples stored at 37°F could be explained by a temperature effect on the chemical reaction(s) occurring. This phenomenon has not previously been reported in processed chlorophyll-containing foods, and was considered to be worthy of further investigation. Extraction, isolation and purification of the pigments responsible, and possible mechanisms for their formation are discussed in Section 3.3.

It is interesting to note that in an examination of the effects of H.T.S.T. processing and pH adjustment on chlorophyll retention in spinach puree (Gupte 1963) it was observed that total pigment concentrations in elevated pH samples decreased markedly on storage up to 6 months at room temperature, but no comment was made as to the significance of these observations. Previously, Tan and Francis (1962) had also noted that in pigment analyses of pH adjusted spinach puree, the analytical data indicated the presence of more pheophytin than could be accounted for by the degradation of chlorophyll. They concluded that some substance

was possibly formed during processing that had higher absorption coefficients than the pheophytins, and was probably present as a contaminant of one of the zones separated by column chromatography. They did not further investigate the cause of the discrepancy.

No attempt was made in this study to determine the effects of H.T.S.T. processing and storage on the content and breakdown of carotenes and xanthophylls. Although leaves and organs of higher plants are known to contain carotenes and at least three xanthophylls, these pigments were not determined since: (1) previous work (Tan and Francis 1962) showed very little change to occur in these pigments compared to the chlorophylls; (2) their determination by spectrophotometry in the region 350-550nm would necessarily involve their chromatographic separation from chlorophylls as the latter also absorb in this region; (3) colour changes due to changes in carotenoid concentration would be small compared to changes involving chlorophyll pigments.

3.234 Summary of Pigment Changes

It is apparent from the foregoing comments on puree processed by H.T.S.T. methods that considerable chlorophyll retention was possible by a combination of processing procedures. The results were in agreement with previous work in this field, and show the possibilities and limitations of attempts to retain bright green pigments in processed materials. The results can be summarised as follows:

1. Increasing the process temperature from 240°F to 300°F of samples at normal pH gave considerable initial retention of chlorophyll pigments.

2. The storage stability of normal pH samples processed by H.T.S.T. methods depended primarily on the storage temperature and storage time. At 68°F at least 86% of the retained chlorophyll was converted within 2 months of storage, while at 37°F, between 41% and 64% was converted within 3 months. Storage at -10°F resulted in little chlorophyll conversion.

3. H.T.S.T. processing of elevated pH samples gave further retention of chlorophylls compared to samples at normal pH. Chlorophyll degradation in conventionally processed samples was considerably reduced by pH elevation, and the storage stability of elevated pH samples, whether processed by conventional or H.T.S.T. procedures, was greatly increased over that of normal pH samples.

4. For elevated pH samples processed by H.T.S.T. methods, storage temperature appeared to be of lesser importance than process temperature in determining the stability of chlorophyll. For normal pH samples, storage temperature was of greater importance than process temperature.

5. Substantial retention of chlorophylls was possible by a combination of high process temperature, elevated pH and low storage temperature.

3.24 Colour Changes in Processed and Stored Pea Puree

The normal and elevated pH pea puree samples prepared previously were analysed for visual colour after storage for periods up to 18 months. Objective colour evaluations were carried out using a Hunterlab Colour and Colour-Difference Meter (Model D25). For repeatable measurements of colour, and for colour differences relative to a colour specified by

numbers, standards are used to keep the instrument scales at their proper numerical 'values'. For this purpose, the instrument had been supplied with five coloured ceramic tiles to which had been assigned L, a and b values in the Hunter-Scofield co-ordinate system. The tiles were checked by reflectance spectrophotometry (Edwards 1963) at the C.S.I.R.O. National Standards Laboratory, Sydney, and L, a and b values calculated from the observed C.I.E. tristimulus values X, Y and Z. (Expt.11.1, Table 4.26). Subsequently, all colour-difference work was carried out using the Hunterlab instrument calibrated against the green tile, with values L = 59.7, a = -16.4, b = 7.4.

The Hunterlab Colour-Difference Meter is designed to measure the colour of flat opaque objects positioned beneath the optical unit. Since transparent or translucent materials would reflect some light from surfaces other than that being measured, it was necessary to establish the minimum cell depth at which all light reflected was from the coloured sample. Consequently, commercially canned peas were pureed, and colour measurements recorded in plastic cells of depth ranging from $\frac{1}{4}$ to $1\frac{1}{2}$ inches (Expt.11.2). The resultant L, a and b values (Table 4.27) showed that constant colour measurements could be obtained using cells at least $\frac{1}{2}$ inch deep, and this practice was adopted throughout the remainder of the colour work described.

3.241 Colour Ranking of Processed Puree

L, a and b values of samples of unprocessed and processed pea puree at normal and elevated pH values were determined by the Hunterlab instrument (Expt.12.1, Table 4.28). The samples were also visually ranked in

order of "desirable green pea colour" by a panel of four judges. The colour indices, together with the previously determined pigment data, are presented in Table 3.09.

Table 3.09 The Relation between Visual Rank, Pigment Content and Colour Indices for Processed Pea Puree

Puree Sample (pH and Process Temperature, °F)	Visual Rank	Percent Chloro-phyll Conversion	Total Chloro-phyll Content (µg/g)	Index $-a/b$	Index $-a/L$	Index $(a^2 + b^2)^{\frac{1}{2}}$
pH6.95, Unprocessed	1	4.0	86.0	0.784	0.443	32.40
pH6.95, 280°F	2	14.6	78.3	0.717	0.404	28.30
pH6.95, 300°F	3	16.3	76.0	0.712	0.398	28.61
pH8.45, Unprocessed	4	4.0	77.2	0.740	0.366	32.61
pH8.45, 300°F	5	10.7	73.0	0.720	0.356	31.65
pH8.45, 280°F	6	8.1	76.4	0.718	0.357	31.89
pH8.45, 260°F	7	15.8	67.3	0.695	0.341	30.32
pH6.95, 260°F	8	30.1	61.8	0.616	0.342	25.73
pH8.45, 240°F	9	34.9	51.4	0.525	0.251	25.18
pH6.95, 240°F	10	83.2	15.5	0.253	0.139	23.21

It should be noted that visual rankings do not attempt to assign a colour score to the samples, but represent the average placement of the samples in order of "desirable green pea colour", with 1 denoting most desirable and 10 denoting least desirable green pea colour. It was interesting to note that, with two exceptions, the samples were ranked in order of decreasing total chlorophyll concentration. The exceptions were normal pH puree processed at 300°F, which was ranked superior in desirable green colour to the unprocessed elevated pH sample, and the elevated pH sample processed at 300°F, which was ranked superior to the sample at the same pH processed at 280°F.

As mentioned previously, elevated pH pea puree was lighter in (green) colour than the normal pH puree due to the incorporation of a suspension of white magnesium carbonate. This was shown quite clearly in the Hunterlab values for the unprocessed purees. Normal pH puree had values L = 45.1, a = -20.0 and b = 25.5, whereas the elevated pH puree, had values L = 52.8, a = -19.4, b = 26.2 (Table 4.28). The largest difference (7.7) was in the L value, but the 'a' and 'b' values showed differences of only 0.6 and 0.7 units respectively. While the two samples were similar in terms of green (-a) and yellow (b) colour values, the normal pH puree was of a darker shade and as a result appeared a somewhat deeper and more natural pea colour. These differences were also illustrated by a comparison of Munsell values of normal and elevated pH puree processed at 300°F. The values were determined by visual comparison with coloured chips from the Munsell Book of Colour (1965). The Munsell notation (Figure 2.8) for the normal pH sample was found to be approximately 5.0GY6/8, while the elevated pH sample was very close in colour to the combination 7.5GY7/8. The elevated pH sample is seen to have a higher "value" or lightness dimension (7 as against 6 for the normal pH sample) and a hue containing less yellow.

The order in which the purees were visually ranked showed that the judges considered the darker colours to be more typical of green peas. Only when the percent chlorophyll conversion of normal pH samples increased (and hence total chlorophyll decreased) to such a level that the green pea colour was overshadowed by the olive green "processed" pea colour typical of commercial products was the lighter colour of the

elevated pH samples considered more desirable (visual ranks 4 to 7).

It should also be noted that while both unprocessed samples showed the same percent chlorophyll conversion (4.0%), their pigment contents measured as total chlorophyll or total pigment were not identical. The high pH puree contained 77.2µg total chlorophyll/g and 80.4µg total pigment/g, while the normal pH puree 86.0µg total chlorophyll/g and 89.6µg total pigment/g. While the presence of magnesium carbonate in the high pH puree would account for the greater part of these differences, since pigment contents were calculated as µg/g puree and not µg/g dry weight, it is possible that some of the pigments at the high pH had been destroyed or were not quantitatively extracted from the puree. The differences in total chlorophyll and total pigment contents between the unprocessed puree samples, despite the identical percent chlorophyll conversion, were also reflected in the colour ratios $^{-a}/b$ and $^{-a}/L$. The ratios for the normal pH puree (0.784 and 0.443 respectively) were higher than the corresponding ratios for the elevated pH puree (0.740 and 0.366 respectively). The larger difference between the values for the ratio $^{-a}/L$ reflected the larger L value of the elevated pH puree.

The colour indices tabulated in Table 3.09 showed that the ratio $^{-a}/L$ was a more consistent indicator of desirable green pea colour than $^{-a}/b$ or $(a^2+b^2)^{\frac{1}{2}}$, i.e. samples were visually ranked in the order of decreasing $^{-a}/L$ value with only two minor exceptions. The elevated pH sample processed at 300°F ($^{-a}/L = 0.356$) was visually ranked superior to the same pH sample at 280°F ($^{-a}/L = 0.357$) although the ratio $^{-a}/b$ of the former (0.720) was slightly higher than that of the latter (0.718).

The 280°F sample also showed a smaller percent chlorophyll conversion (and hence larger total chlorophyll) and a smaller value for the index $(a^2+b^2)^{\frac{1}{2}}$. The elevated and normal pH samples processed at 260°F, visually ranked 7 and 8 respectively, gave $^{-a}/L$ values of 0.341 and 0.342 respectively. The order of visual ranking for the two samples, however, was in agreement with the results for percent chlorophyll conversion, total chlorophyll content, ratio $^{-a}/b$ and index $(a^2+b^2)^{\frac{1}{2}}$.

It can be observed that for the last five samples visually ranked from 6 to 10 in terms of desirable green pea colour, the rank order was in agreement with pigment results (percent chlorophyll conversion or total chlorophyll) and colour indices ($^{-a}/b$, $^{-a}/L$ and $(a^2+b^2)^{\frac{1}{2}}$) with the one minor exception (ratio $^{-a}/L$) noted previously. The relationship between visual rank order and objective measurements for the other five samples was less clear, apparently because of the small differences in numerical results between the samples. It is apparent that despite the exceptions noted, there was overall agreement between the results for visual ranking, pigment content and colour measurements, and this was evidenced by the significant correlation coefficients found relating subjective rankings to objective determinations as given in Table 3.10.

Table 3.10 Relation between Visual Rank Score, Pigment Content and Colour Indices for Processed Pea Puree

Relationship	Correlation Coefficient "r"
Visual Rank vs Total Chlorophyll	-0.854
Visual Rank vs Percent Chlorophyll Conversion	0.741
Visual Rank vs Index $^{-a}/b$	-0.789
Visual Rank vs Index $^{-a}/L$	-0.880
Visual Rank vs Index $(a^2+b^2)^{\frac{1}{2}}$	-0.693

It was of interest to compare the rank order of unprocessed and processed puree samples considered together (Table 3.09) with the order of ranking of samples separately within each pH group. Consequently, samples of normal or elevated pH were ranked in order of "desirable green pea colour", and it was found that the rank order within each pH group could be predicted from Table 3.09. For the normal pH samples, there was substantial agreement between visual rank and the various pigment and colour indices with the exception of the index $(a^2+b^2)^{\frac{1}{2}}$, for which the 280°F sample (28.30) was ranked superior to the 300°F sample (28.61).

For the elevated pH samples, visual rank did not correlate completely with pigment and colour indices with the exception of the colour index $^{-a}/_{b}$. Correlation coefficients relating visual rank score with various pigment and colour indices for puree samples of different pH are given in Table 3.11. Coefficients relating determinations on normal pH samples were in each case higher than the corresponding results for elevated pH samples, apparently due to the incorporation of magnesium carbonate as a white suspension.

Table 3.11 Relation between Visual Rank Score, Pigment Content and Colour Indices for Processed Pea Puree at Normal and Elevated pH Values

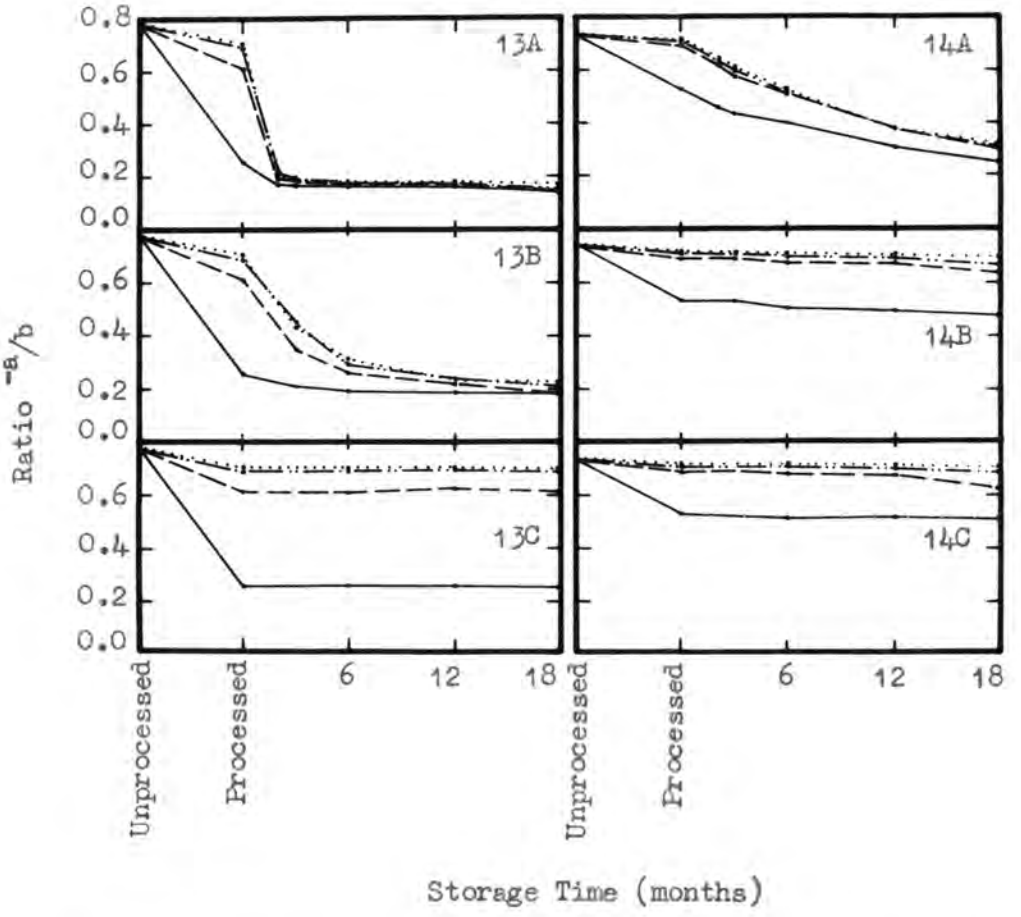
Relationship	Correlation Coefficient "r"	
	Normal pH	Elevated pH
Visual Rank vs Total Chlorophyll	0.905	0.814
Visual Rank vs Percent Chlorophyll Conversion	0.877	0.876
Visual Rank vs Index $^{-a}/_{b}$	0.869	0.820
Visual Rank vs Index $^{-a}/_{L}$	0.877	0.825

3.242 Effect of Processing and Storage

Table 3.09 shows the effects of process temperature and pH on the change in colour of pea puree as measured by colour indices derived from L, a, b data. The results agree closely with pigment changes during processing (Table 3.03) and show that quantitative colour measurements give a reasonably good indication of chlorophyll pigment changes in processed green foods.

Colour changes during storage (Tables 4.29 to 4.40) followed very closely the changes in pigments discussed previously (Tables 3.05, 3.06). When the hue ratio $^{-a}/_{b}$ was plotted against storage time for each process temperature, storage temperature and pH, the resulting curves (Graphs 13 and 14) were very similar to the corresponding total chlorophyll curves (Graphs 10 and 11). Any green colour retained as a result of a H.T.S.T. process (260°F, 280°F and 300°F) was rapidly lost during the first 2 months of storage at 68°F, or 6 to 12 months at 37°F for puree of normal pH. Colour changes during storage of elevated pH puree at 68°F were less rapid than colour changes in normal pH puree at 37°F. Storage of normal pH puree at -10°F, or elevated pH puree at 37°F or -10°F, showed minor changes in colour as measured by any colour index. Thus colour stability during the storage of H.T.S.T. processed puree of normal pH was several days at 68°F, several weeks at 37°F, and possibly years at -10°F. For elevated pH puree, colour stability amounted to months at 68°F storage, and years at 37°F or -10°F storage.

From observations such as these, it was apparent that adjustment of the pH of the product was a very useful method for retaining green



Effect of processing and storage on the Hunterlab ratio $^{-a}/_{b}$ of pea puree.

Graph 13 Normal pH

Graph 14 Elevated pH

A	Storage temp.	68°F	———	Process temp.	240°F
B	" "	37°F	- - -	" "	260°F
C	" "	-10°F	- . . -	" "	240°F
			.....	" "	300°F

colour. When combined with an H.T.S.T. process and refrigerated storage such methods would result in a product with a much prolonged colour stability.

It was apparent from the similar results for pigment and colour changes during processing and storage that a significant relationship existed between the two, and accordingly, correlation coefficients relating percent chlorophyll conversion and colour indices $^{-a}/_{b}$, $^{-a}/_{L}$ and $(a^2+b^2)^{\frac{1}{2}}$ were calculated by computer at the C.S.I.R.O. Division of Computing Research, Canberra. The results (Table 3.12) show that an almost statistically perfect relationship existed between percent chlorophyll conversion and the colour indices $^{-a}/_{b}$ and $^{-a}/_{L}$ for normal pH puree. It is seen that these two ratios changed in an almost identical manner and either could be used interchangeably to estimate percent chlorophyll conversion in samples as used in this work.

Table 3.12 Relation between Percent Chlorophyll Conversion and Colour Indices for Processed Pea Puree at Normal or Elevated pH

Relationship	Correlation Coefficient "r"	
	Normal pH	Elevated pH
Percent Chlorophyll Conversion vs Index $^{-a}/_{b}$	-0.997	-0.962
Percent Chlorophyll Conversion vs Index $^{-a}/_{L}$	-0.995	-0.950
Percent Chlorophyll Conversion vs Index $(a^2+b^2)^{\frac{1}{2}}$	-0.888	-0.951
Ratio $^{-a}/_{b}$ vs $^{-a}/_{L}$	0.999	0.993

Correlations between indices for elevated pH puree were lower than for normal pH puree with the exception of chlorophyll conversion vs $(a^2+b^2)^{\frac{1}{2}}$. Since the index $(a^2+ b^2)^{\frac{1}{2}}$ approximates to the saturation of a colour, it

is apparent that changes in saturation are of greater significance in lighter colours (i.e. elevated pH puree) than in darker colours, whereas changes in hue, e.g. green to yellow, as measured by $^{-a}/_{b}$ ratio or $^{-a}/_{L}$ ratio appeared satisfactory for both dark and light colours, but were very highly significant for dark colours.

The close relationship between percent chlorophyll conversion and the ratio $^{-a}/_{b}$ is shown in Graph 15 for samples at the two pH values. For normal pH puree, the regression equation calculated was:

Ratio $^{-a}/_{b}$ = 0.8115 - 0.0065 (percent chlorophyll conversion)

with a standard error S = 0.0169 for the estimation of $^{-a}/_{b}$.

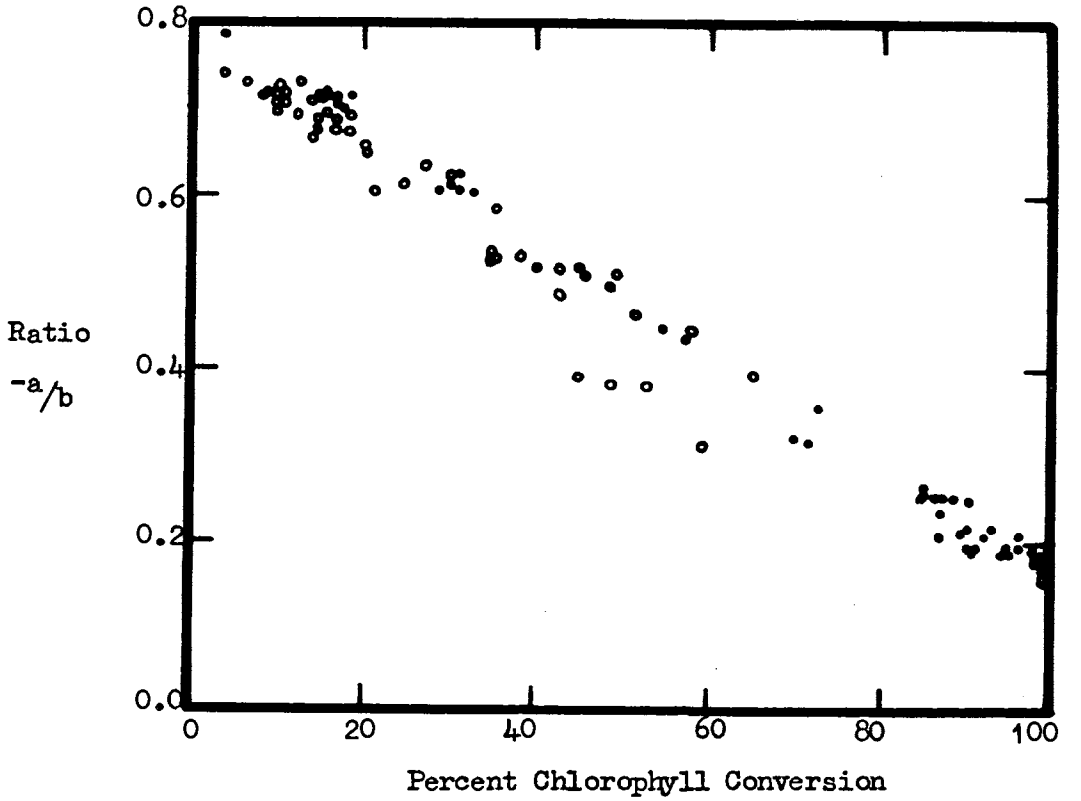
For elevated pH puree, the regression equation was:

Ratio $^{-a}/_{b}$ = 0.7816 - 0.0066 (percent chlorophyll conversion)

with a standard error S = 0.0321.

3.25 pH Changes in Processed and Stored Pea Puree

Changes in pH as a result of processing (Expt. 13, Table 4.41) were in agreement with expected trends in that irrespective of initial pH, lower process temperatures produced a much greater pH change than higher temperatures. The pH changes for normal pH puree were greater, however, than the changes for elevated pH samples processed at corresponding temperatures. Within each pH variable, changes in pigment and colour for each process temperature were proportional to the pH change during processing. The large decrease in pH for samples heated by a conventional process (i.e. 240°F) would account for the substantial conversion of chlorophyll to pheophytin, since the rate of this reaction is dependent on the acid concentration. Since the action of organic acids from



GRAPH 15

Relationship between the ratio $^{-a}/_{b}$ and percent chlorophyll conversion for processed and stored pea puree.

● Normal pH

o Elevated pH

ruptured cells is held to be intimately connected with the degradation of pigments with conventional processes, it is apparent that adequate buffering or the use of alkaline media may retard such pH changes and hence result in initial chlorophyll and colour retention.

The changes in pH during storage were affected by storage temperature and storage time, and showed trends similar to those observed for chlorophyll pigments and colour. During storage for 18 months at 68°F, the pH of normal pH puree processed at 240°F decreased by only 0.38 units while puree processed at 260°F, 280°F and 300°F decreased by 0.66, 0.76 and 0.86 pH units respectively. The changes during the first 2 months storage were 0.26, 0.35, and 0.54 pH units for 260°F, 280°F and 300°F samples respectively, and represented the largest single changes throughout the whole storage period of 18 months. These results were in agreement with the pigment and colour changes observed during storage of normal pH samples at 68°F and showed that these pH changes were primarily responsible for the rapid conversion of pigment and resulting change of colour.

The pH changes in elevated pH puree stored at 68°F were smaller than the changes in normal pH samples. The pH changes in samples processed at 240°F, 260°F, 280°F and 300°F and stored for 18 months at 68°F were 0.46, 0.46, 0.45 and 0.39 respectively, the order of magnitude of the changes being the reverse of that found for samples of normal pH puree. From these results it would appear that in normal pH samples, the decrease in pH during processing was proportional to the heat treatment given, but the rate of pH change during storage at 68°F was dependent on the differ-

ence between the pH after processing, but before storage, and the "ultimate pH" of the product. The latter value is suggested as being typical of a particular food material processed and stored under given conditions, and dependent on the original constitution of the material with respect to organic acids, buffering capacity and other factors that would influence an "ultimate" or equilibrium pH value.

The pH change in elevated pH puree was also proportional to the severity of the heat process, but the changes during storage were similar for each process temperature and were presumably determined by the high initial pH and conditioned by the buffering capacity of the alkaline puree.

It was of interest to note that while a H.T.S.T. process produced smaller pH changes during processing than a conventional process irrespective of initial pH, the pH of H.T.S.T. samples decreased more rapidly than commercially processed samples for normal pH puree, but less rapidly for elevated pH samples. The one exception was normal pH puree processed at 240°F, which showed a smaller pH change in storage than any other sample due to the large decrease in pH that had occurred during processing. Results of pH changes during H.T.S.T. processing and storage of spinach puree (Clydesdale 1966) were in agreement with the different rates of pH change between conventional and H.T.S.T. processes observed in the present investigation, but were largely different in other respects.

Clydesdale found that (1) conventional processing of normal pH (initial pH6.70) and elevated pH (initial pH8.65) spinach puree resulted in a decrease in pH of 0.65 and 0.95 units respectively, and storage at

room temperature (75°F) for 6 months resulted in a further decrease of 0.14 and 0.50 units respectively. The present work on pea puree showed a much greater pH decrease in the normal pH sample than in the elevated pH sample after a process at 240°F; (2) identical samples processed by a H.T.S.T. method (300°F) showed very small pH changes due to processing (zero and 0.20 pH units for normal and elevated pH puree), but storage for 6 months at room temperature resulted in pH decreases of 0.80 and 1.65 pH units respectively. This latter result, i.e. the greater pH change in the elevated pH sample processed by a H.T.S.T. method, is in direct contrast to the results found in the present work, where the pH change during storage of elevated pH pea puree processed at 300°F (0.39 pH units) was much smaller than the corresponding change in the normal pH sample during storage for 18 months (0.86 pH units). An explanation of this phenomenon could be the much greater buffering capacity of peas compared to spinach. This supposition is substantiated by the fact that 0.35% magnesium carbonate powder was required to elevate the pH of spinach puree from pH6.70 to 8.65 (Clydesdale 1966) whereas more than 2% was required to elevate the pH of blanched pea puree from 6.95 to 8.45 in the present study.

3.26 Summary of Changes in Stored Processed Pea Puree

The results from this study show that chlorophyll pigment changes during processing and storage can be closely followed by tristimulus colour measurements. Hunterlab colour indices $^{-a}/_{b}$ and $^{-a}/_{L}$ very closely correlated with total chlorophyll or percent chlorophyll conversion in processed samples during 18 months storage at temperatures from 68°F to

-10°F. With the use of regression equations similar to those discussed previously, pigment data could be calculated from Hunterlab indices providing conditions of analysis were standardised as in this investigation.

The incorporation of magnesium carbonate into puree resulted in Hunterlab values slightly different to normal pH puree of identical chlorophyll conversion and consequently colour measurements could be used to estimate pigment composition of processed samples so long as due regard were taken of such differences.

Pigment and colour changes during processing and storage followed closely the changes in pH of puree samples. Large decreases in pH were accompanied by conversion of chlorophylls with a consequent formation of unattractive colours. Changes in pH were greater for samples of low initial pH processed at low temperatures, and were accelerated by storage at high temperatures for long periods. Chlorophyll retention, therefore, was increased by either high initial pH, high process temperatures or low storage temperatures, and a combination of two or more of the variables produced samples of acceptable colour with a greatly enhanced storage life.

3.3 ISOLATION AND PURIFICATION OF PIGMENTS FROM H.T.S.T. PROCESSED PEA PUREE

It was mentioned previously (Section 3.233) that pigment determinations on H.T.S.T. processed elevated pH pea puree stored for 18 months at 68°F and 37°F were in error particularly in respect to the percent conversion of total chlorophyll and chlorophyll **a**, and in the concentrations of the **a** pigments as shown by the change in the ratio of total a/b

pigments. Examination of the gross pigment extracts from normal and elevated pH puree stored 18 months at 68°F (Graph 12) indicated the presence in the elevated pH extract of a pigment or several pigments with spectra grossly different to those of the chlorophyll pigments normally found in heat processed foods (i.e. chlorophylls a and b and pheophytins a and b). Since pigment extracts from H.T.S.T. processed foods had not previously shown the unusual spectra found in this work, it was decided to isolate and purify all pigments in the extracts from both normal and elevated pH puree, and to characterise the pigment(s) responsible for the unusual spectra.

Subsequently, an examination was made of the literature on the chromatographic separation of low concentrations of chlorophyll-type pigments, in particular by thin-layer chromatography. Reports describing the use of cellulose powder (Bacon 1964,1965a,b; Bacon and Holden 1967a), powdered sugar (Colman and Vishniac 1964; Nutting et al.1965) and other adsorbents were examined and the methods thoroughly tested using crude and purified chlorophyll and pheophytin preparations. The use of silica gel plates similar to those recently described by Lynn and Schanderl (1967b) were found to give poor separations and to produce degradation products from pure pigments, in agreement with the work of Bacon (1966, 1967) and were not further considered in this study. Powdered sugar plates, while not producing any detectable artifacts, were not as satisfactory as cellulose plates with respect to development time and pigment resolution, and consequently the method of Bacon (1965a,b) and Bacon and Holden (1967a) was adopted with some minor modifications (Expt.14).

The adsorbent was spread on to glass plates at a thickness of approximately 0.5mm, about twice the thickness of normal thin layers. Preliminary experiments had shown that layers of this thickness could separate larger quantities of pigments without loss of resolution. Plates prepared with layer thicknesses greater than 0.5mm were found to show severe cracking of the adsorbent after oven drying. Pigment solutions in ether were applied to the plates using the apparatus described by Monteiro (1965). This enabled a considerable quantity of pigment to be applied to a single plate as a streak along the baseline. If the solvent was allowed to evaporate between each application, a narrow pigment band was formed. The pigment band was further concentrated, when necessary, by dipping the edge of the plate into a trough of acetone until the solvent front was just above the baseline of the chromatogram (Bacon and Holden 1967a).

To avoid the occurrence of "edge" effects in the separation of the pigments, the adsorbent on both sides of the plate was scraped away about 2mm from the edge. Since this necessarily meant that some of the pigment band was also removed, quantitative analysis of pigments using the above modifications was not possible. In addition, as the absorption coefficients of a number of the separated pigments were unknown, their concentrations could not be calculated from absorbance determinations. Instead, relative amounts of the different pigments were estimated by the size of the pigment band and its fluorescence under UV light, and a comparison with absorbances of solutions containing known pigments.

3.31 Pigments from Elevated pH Pea Puree

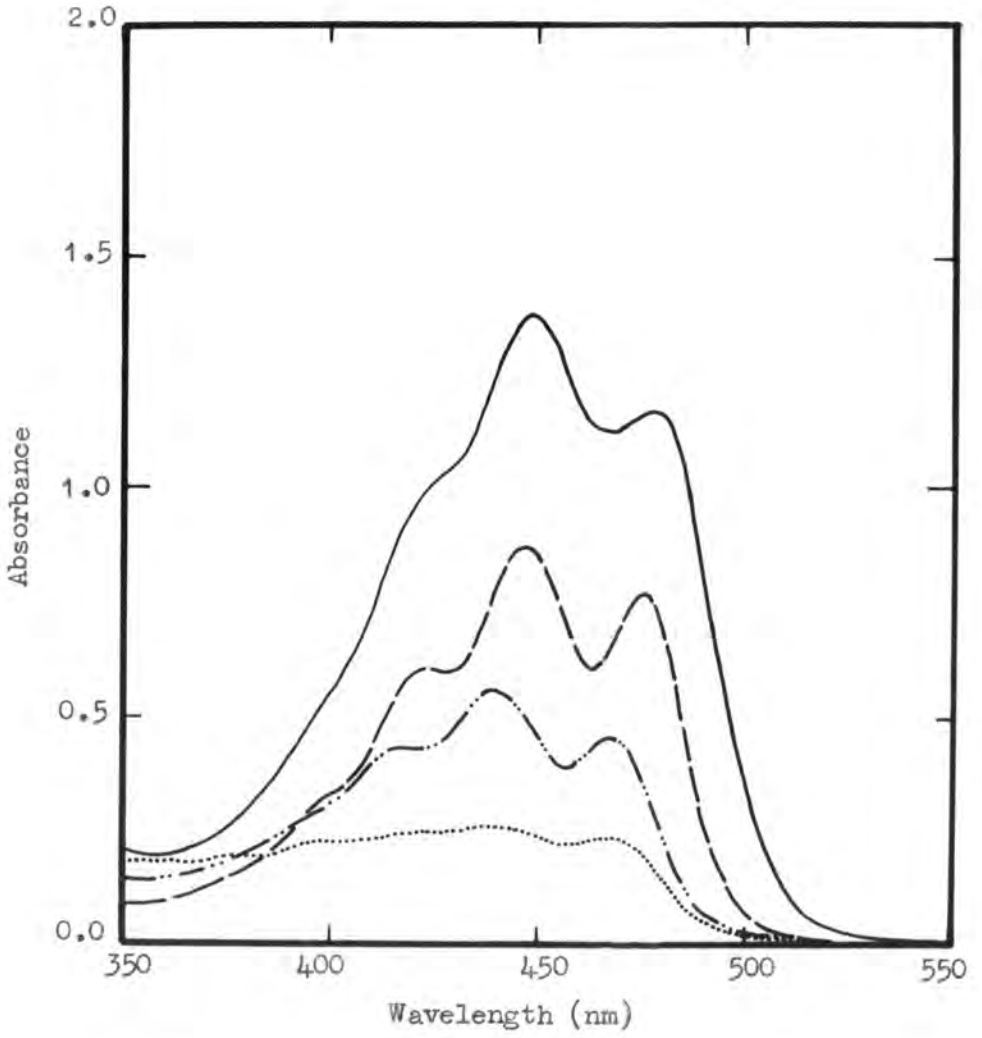
Pigments were extracted from puree processed at 300°F after storage

for 18 months at 68°F. The pigment extract was chromatographed on thin layers of cellulose powder (Expt.15.1), and when examined in daylight and under UV light, 12 reproducible pigment bands of various colour were observed (Table 4.42). The bands were scraped off the plates, eluted with acetone, and rechromatographed on columns or thin-layers. Fraction E1 was the least adsorbed (travelled furthest) and fraction E12 the most adsorbed (travelled least). The spectral absorption characteristics are summarised in Table 4.43.

1. Fraction E1 was yellow under visible light and bluish-white under UV, and travelled with the solvent during development. Rechromatography was carried out on the more polar adsorbent aluminium oxide, and the purified pigment showed absorption maxima at 424,450 and 478nm in n-hexane (Graph 16) and at 424,448 and 476nm in petroleum ether (b.p.40-60°C). The absorption maxima were similar to those reported in the literature for β-carotene (Karrer and Jucker 1950; Goodwin 1955) and this identification was consistent with its chromatographic behaviour (Strain 1958).

2. Fraction E2, a grey pigment slightly more adsorbed than β-carotene, showed maxima at 409,470,506,534,562,609 and 666nm in diethyl ether (Graph 17). These absorption maxima were almost identical to those found previously for pheophytin a and the pigment showed the same chromatographic and chemical behaviour. This pigment was present in the highest concentration of those isolated.

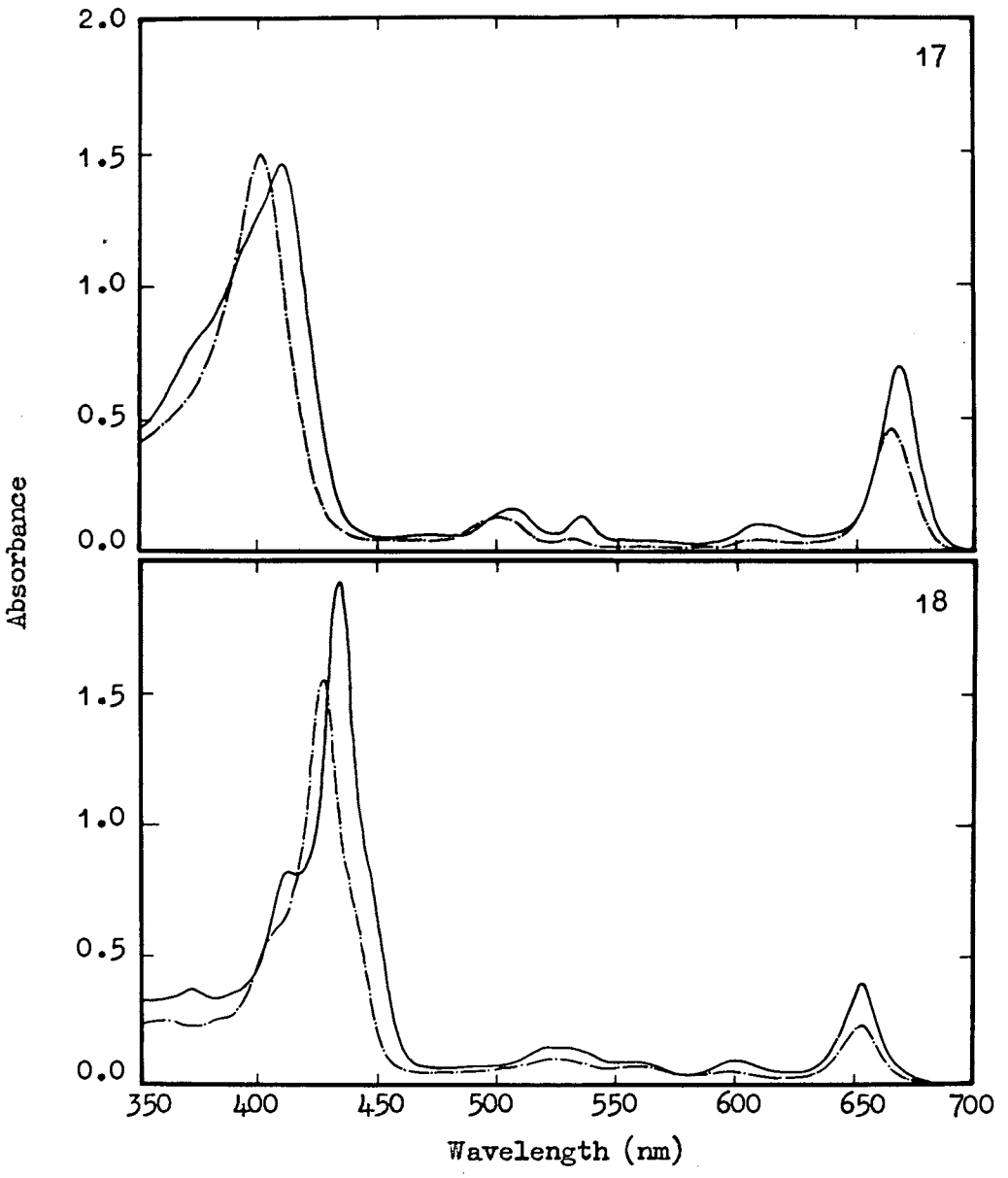
3. Fraction E3, a yellow band fluorescing green under UV light, was freed from pheophytin contamination on a sugar/cellulose column and



GRAPH 16

Spectral curves of pigments from elevated pH pea puree.

——— E 1 β-carotene (n-hexane)
••••• E 3.1 unidentified (P.E.40°-60°C) (40mm)
- ·· - E 3.2 unidentified (n-hexane)
– – – E 3.3 lutein (n-hexane)



Spectral curves of pigments from elevated pH pea puree.

Graph 17	———	E 2	pheophytin a (diethyl ether)
	-·-·-	E 5	pigment X (" ")
Graph 18	———	E 4.2	pheophytin b (diethyl ether)
	-·-·-	E 7.1	pigment Y (" ")

subsequently chromatographed on alumina. Initial development with up to 10% acetone in petroleum ether (b.p.40-60°C) resulted in the separation of a colourless, strongly blue fluorescent band from the major coloured zone. The colourless band travelled with the solvent front under these conditions, and was not collected. Many colourless polyenes in pigment extracts from higher plants have been characterised (Goodwin 1955) and undoubtedly this fraction was a pigment of this nature.

On continued development, the yellow band separated into two minor yellow fractions (E3.1,E3.2) and a major yellow band (E3.3). The lower, pale yellow fraction E3.1, fluoresced pale green under UV light and showed indistinct absorption maxima in petroleum ether (b.p.40-60°C) at approx. 398,422,439 and 468nm (Graph 16.). The upper pale lemon-yellow band (E3.2), absorbed more strongly on the column, showed absorption maxima at 418,439 and 468nm in n-hexane (Graph 16) and was present at approximately six times the concentration of E3.1.

The uppermost major golden yellow band (E3.3) showed maxima at 422, 446 and 476nm in n-hexane (Graph 16), and at 420,445 and 474nm in petroleum ether (b.p.40-60°C). The results in n-hexane were in close agreement to the values (420,447,477nm) reported by Goodwin (1955) and others for the xanthophyll lutein (3,3'-dihydroxy-α-carotene). The chromatrographic behaviour of this pigment on mild adsorbents such as sugar and cellulose (Strain 1958; Bacon and Holden 1967a) was also in agreement with this identification.

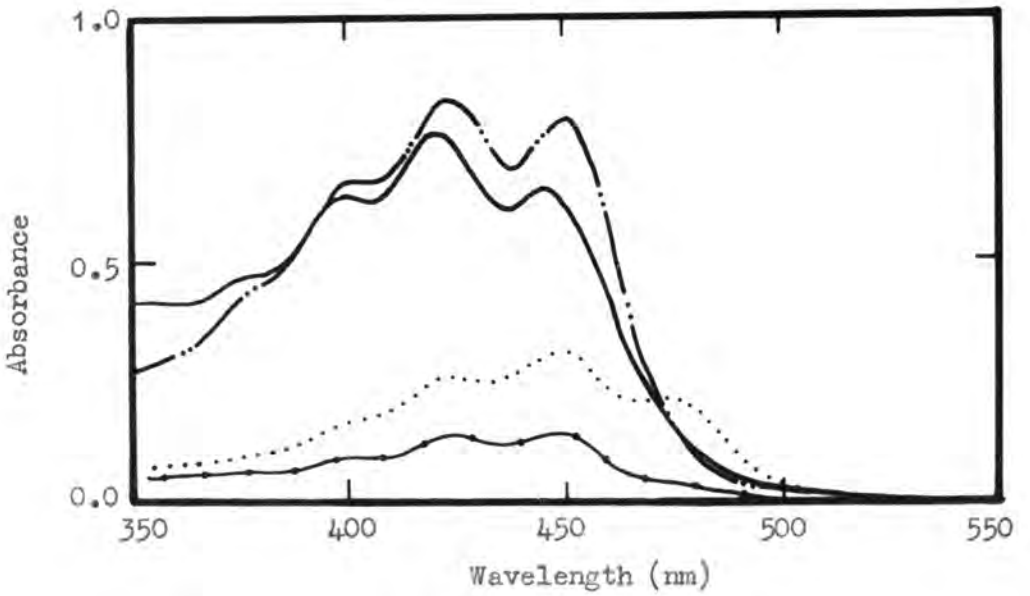
Identification of the minor components E3.1 and E3.2 was difficult because of their low concentrations, but it was possible they were de-

gradation products of lutein formed during the chromatography on alumina. Their similar absorption spectra showed evidence of similar structures, but they were not identified further.

4. Fraction E4, yellow-green and showing red fluorescence under UV separated into two bands (E4.1, E4,2) when chromatographed on sugar/cellulose. The minor, least-adsorbed greenish-yellow band (E4.1) was chromatographed on alumina, and upon development two colourless bands separated from the major pigment fraction. Both bands fluoresced green under UV, but were not collected. The coloured band finally separated into two yellow bands on further development. The lower band (E4.11) showed maxima at 400,421 and 447nm in petroleum ether (b.p.40-60°C), while the uppermost, more strongly adsorbed band (E4.12) showed maxima at 403,424 and 451nm in the same solvent (Graph 19). The identity of bands E4.11 and E4.12 was not established, because of the small quantities of pigments available and because the spectral maxima were obviously not responsible for the unusual spectra of the gross extract. They were probably breakdown products of one or more of the constituent carotenoids.

Fraction E4.2, yellow-green, was rechromatographed on sugar/cellulose and showed absorption maxima in diethyl ether at 412,434,524,556,600 and 654nm (Graph 18). The pigment was identified as pheophytin b by comparison with previous results and with its position on the thin-layer plate (Bacon 1965a).

5. Fraction E5, grey under visible light and a strong fluorescent red under UV, was chromatographed on sugar/cellulose and cellulose and showed absorption maxima at 401,502,531,560,609 and 664nm in diethyl ether



GRAPH 19

Spectral curves of pigments from elevated pH pea puree.

———	E 4.11	unidentified (P.E. 40°-60°C) (40mm)
-·-·-	E 4.12	unidentified (P.E. 40°-60°C) (40mm)
·····	E 7.2	violaxanthin? (acetone)
·-·-·-	E 9	unidentified (acetone) (40mm)

(Graph 17) and at 400,501,529,558,607 and 662nm in acetone. This pigmentwas present at a slightly lower concentration than band E2 (pheophytin a), and on initial inspection showed an absorption spectrum somewhat similar to pheophytin a. However, there were a number of important differences between the spectra of the two pigments (Graph 17).

(1) The absorption maximum in the red region of fraction E5 (664nm) was shifted towards the blue compared to that of pheophytin a (666-667nm) in diethyl ether.

(2) The absorbances of peaks between 500nm and 650nm for fraction E5 were lower relative to the corresponding peaks in pheophytin a.

(3) The ratio of the peak heights in the region near 500-534nm was significantly higher in fraction E5 than in pheophytin a because of the greatly reduced height of the absorption peak near 530nm.

(4) The major blue absorption maximum (401nm) of fraction E5, although of comparable intensity to the peak in pheophytin a, (409nm), was shifted towards the blue and failed to show the typical "bumps" on the curve below 400nm characteristic of the pheophytin a spectrum.

(5) The ratio of the blue/red absorbance was greater for fraction E5 than for pheophytin a.

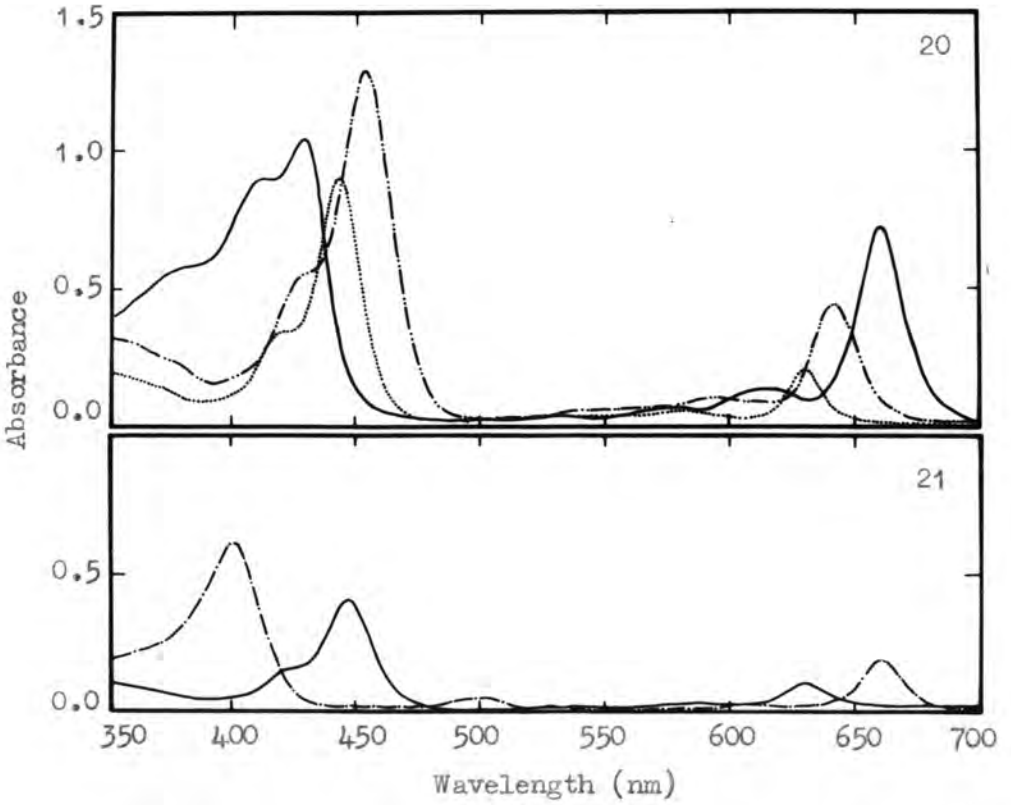
These differences between the two spectra were found to correspond closely to some of the discrepancies observed in the total pigment extract from elevated pH puree compared to that from normal pH puree. From these results it was obvious that the presence of fraction E5 was totally or largely responsible for the erroneous pigment determinations. The pigment

was designated pigment X and was subsequently analysed in greater detail (Expt.17, Section 3.33).

6. Fraction E6 (blue-green) was present in only low concentration, and after rechromatography by T.L.C. showed absorption maxima in diethyl ether at 410,429,500,533,577,615 and 660nm, which were identical with those found previously for chlorophyll a (Table 4.02). The absorption spectrum (Graph 20) was slightly different, however, to that previously observed (Graph 1). It was noticed that the height of the major blue peak (429nm) was reduced considerably compared to that of the minor blue peak (410nm). This could be due either to a slight chemical alteration of the pigment structure, or possibly to the presence of an impurity which was not separated under the conditions of chromatography used in this work. Since pigments with similar spectra were sometimes isolated from "purified" chlorophyll a samples which had been allowed to stand in organic solvents for lengthy periods, even at -20°F, it would appear that a minor chemical or structural change had occurred.

7. Fraction E7 (yellow-green) was separated into two fractions by chromatography on sugar/cellulose (E7.1,E7.2). The lower,major greenish-yellow band E7.1 (red under UV), subsequently designated pigment Y, showed absorption maxima at 427,524,561,599 and 653nm in diethyl ether (Graph 18), and at 426,523,559,596 and 651nm in acetone. Comparison of the spectrum in diethyl ether to that of fraction E4.2, pheophytin b (Graph 18) showed overall similarity, but a number of important differences were also apparent. These were:

(1) The red absorption maximum(653nm) of fraction E7.1 was similar



Spectral curves of pigments from elevated pH pea puree.

Graph 20	———	E 6	chlorophyll a (diethyl ether)
	-·-·-	E 8	chlorophyll b (" ")
	·····	E 10	pigment Z (" ")
Graph 21	———	E 11	unidentified (acetone) (40mm)
	-·-·-	E 12	unidentified (") (")

to that of pheophytin b.

(2) The absorbances of peaks between 500nm and 650nm in E7.1 were considerably lower than the corresponding peaks of pheophytin b.

(3) The blue absorption maximum (427nm) was shifted toward lower wavelengths compared to pheophytin b (434nm).

(4) The secondary blue absorption peak in the pheophytin b spectrum (412nm) was reduced to a "bump" or inflection at approximately 406nm.

(5) The ratio of blue/red absorbance was greater for fraction E7.1 than for pheophytin b.

It is at once obvious that the differences described above are similar to those observed between the spectra of pheophytin a and pigment X (fraction E5). Although pigment Y and pheophytin b were present in approximately equal concentrations, they were present together at less than half the total concentration of pheophytin a plus pigment X, and consequently the spectrum of pigment Y was not obvious in an examination of the total pigment spectrum (Graph 12). However, the presence of pigment Y would also result in erroneous pigment determinations due to differences in absorption coefficients. The properties of pigment Y were examined in greater detail (Expt.17) and are discussed in Section 3.33. Fraction E7.2 (yellow) showed maxima at 423,449 and 474nm in acetone (Graph 19). Although adequate identification could not be achieved, this pigment may have been the xanthophyll violaxanthin (3,3'-dihydroxy-5,6,5',6'-diepoxy-β-carotene) since it has been well established (Strain 1958; Strain et al.1965,1968) that this carotenoid is

present in all higher plants which undergo photosynthesis. Bacon and Holden (1967a) reported that violaxanthin travelled approximately as far as chlorophyll a, but their conditions of chromatography may have been slightly different to those reported here.

8. Fraction E8 (green) showed absorption maxima at 430,454,548,569,594 and 642nm in diethyl ether (Graph 20). The pigment was identified as chlorophyll b by its absorption spectrum, colour, and chromatographic behaviour. The absorption spectrum was slightly different to that found previously (Graph 2) in that the secondary blue maximum (430nm) was not as sharp and appeared more as an inflection than as a distinct absorption peak. This was previously found in pigment Y when compared to the spectrum of pheophytin b.

9. Fraction E9 (pale yellow) showed indistinct maxima at 400,423 and 450nm in acetone (Graph 19) after T.L.C. on cellulose. As the carotenoid neoxanthin (3,3',5'-trihydroxy-6'hydro-5,6-epoxy-β-carotene) has been shown to be present with β-carotene, lutein and violaxanthin in photosynthetic tissues of most higher plants (Strain 1958), it is possible that fraction E9 was a derivative of neoxanthin, possible the furanoid derivative neochrome, which has been shown to be produced from neoxanthin under various chromatographic conditions and shows similar spectral maxima (400,422,448nm) in ethanol (Strain et al.1967). Insufficient material was isolated, however, to attempt a positive identification.

10. Fraction E10 formed a green band when viewed in daylight, but displayed a distinctive orange-red fluorescence under UV light. After rechromatography the pigment showed absorption maxima at 420,444,536,582

and 630nm in diethyl ether (Graph 20). The overall shape of the absorption curve was similar to that of chlorophyll b, but some important differences were observed.

(1) The red absorption maximum in diethyl ether (630nm) was shifted considerably towards the blue compared to chlorophyll b (642-643nm).

(2) The major blue peak (444nm) was also shifted towards the blue from that of chlorophyll b (454nm). Secondary maxima were also displaced.

(3) The blue/red absorbance ratio was greater in E10 than in chlorophyll b.

These differences between fraction E10, subsequently designated pigment Z, and chlorophyll b were similar to those previously observed between pheophytin a and pigment X (E5) and between pheophytin b and pigment Y (E7.1). The amount of pigment Z present in the gross extract appeared to be greater than that of chlorophyll b, but considerably less than pigment X or pigment Y. Further examination of pigment Z (Expt.17) is discussed in Section 3.33.

11. Fraction E11 was present in very low concentration and appeared to be a faint green in colour. The absorption maxima in acetone were at 422,446,582 and 630nm (Graph 21), similar to those determined for pigment Z. Further characterisation was not possible because of the trace amount of the pigment.

12. Fraction E12 was distinctly visible only under UV light, and had travelled less than 2cm during development. The absorption maxima in acetone (400,500,530,560,617,661nm, Graph 21) were similar to those previously determined for pigment X, although the positions of the pigments

on the plate showed that they were compounds of different polarity. Further examination was not possible because of a lack of pigment.

The isolation of unusual chlorophyll-type pigments from elevated pH puree stored at 68°F would tend to indicate that formation of these pigments had been caused by chemical reactions due to the alkaline pH of the puree. To observe whether such changes had occurred at low temperatures or at a normal pH comparable to commercially canned foods, pigments from puree stored under these conditions were also analysed by cellulose thin-layer chromatography.

The pigments isolated and identified (in order of increasing absorption on a cellulose T.L.C. plate) from pH-elevated puree stored 18 months at -10°F (Expt.15.2, Table 4.44), were β-carotene, pheophytin a, lutein, (pheophytin b), chlorophyll a', chlorophyll a, chlorophyll b' and chlorophyll b. Band E16 was present in insufficient quantity for spectral analysis, but its position on the plate would indicate that it was pheophytin b. The low concentration present was in agreement with previous pigment determinations (Table 4.25). The pale yellow zone (E21) showed similar chromatographic behaviour to fraction E9, but was not characterised further.

Narrow blue-green and green bands running just ahead of chlorophyll a and chlorophyll b were identified as chlorophyll a' and chlorophyll b' respectively by their similar spectra to the parent compounds, and their slightly faster chromatographic separation (Strain 1954; Pennington et al.1964). It has been postulated that chlorophyll a' and chlorophyll b' are optical isomers at C-10 of chlorophyll a and chlorophyll b.

Pigments with absorption spectra similar to pigments X, Y or Z were not found in puree stored at -10°F, showing that their formation was not caused by the chromatographic procedure. The observation that chromatography of purified pigments X, Y and Z or of purified pheophytin a, pheophytin b or chlorophyll b did not produce any detectable degradation products also demonstrated that the pigments isolated from puree extracts were initially present and not artifacts of the method of isolation.

3.32 Pigments from Normal pH Pea Puree

The absorption maxima of pigments isolated from normal pH puree stored 18 months at 68°F are shown in Table 4.45. The pigments β-carotene, pheophytin a, lutein and pheophytin b were found to be the principal compounds present, with pheophytin b at a concentration less than half that of pheophytin a. Lutein was present at a higher concentration than β-carotene. Small amounts of two compounds (fractions N5 and N7)with similar chromatographic behaviour and absorption spectra to pigments X and Y respectively were isolated from the extract, but their combined concentration was estimated at less than 5% of the total pheophytin concentration. This indicated that the degradation reactions leading to the formation of pigments X and Y were possible under the conditions of storage, but were greatly accelerated by an alkaline pH and retarded by low temperatures.

Minor yellow pigments separated (fractions N6 and N8) could not be spectrally identified,but their chromatographic behaviour would indicate that they were probably violaxanthin and neoxanthin respectively (Bacon and Holden 1967a). The absence of chlorophylls a and b was in agreement with previous pigment determinations (Table 4.17).

Pigments isolated from normal pH pea puree stored for 18 months at -10°F (Table 4.46) were found to be identical to those found in high pH puree stored at the same temperature (Table 4.44) with the exception that a faint yellow band (N13) was present between pheophytin b and chlorophyll a'. This pigment showed different chromatographic behaviour to fraction N5 and was probably not identical. Pigments similar to pigments X, Y or Z were not found.

3.33 Characterisation of Unusual Pigments from Processed Pea Puree

Chromatographic separation of pigments from processed pea puree showed the presence of a number of compounds with absorption spectra different from any of the usual chlorophyll-derived pigments previously found as degradation products in heat processed foods. These compounds altered the shape of the gross pigment spectrum (Graph 12) and resulted in erroneous pigment determinations by spectrophotometry, and consequently an attempt was made to characterise the pigments in terms of their spectral and chemical properties.

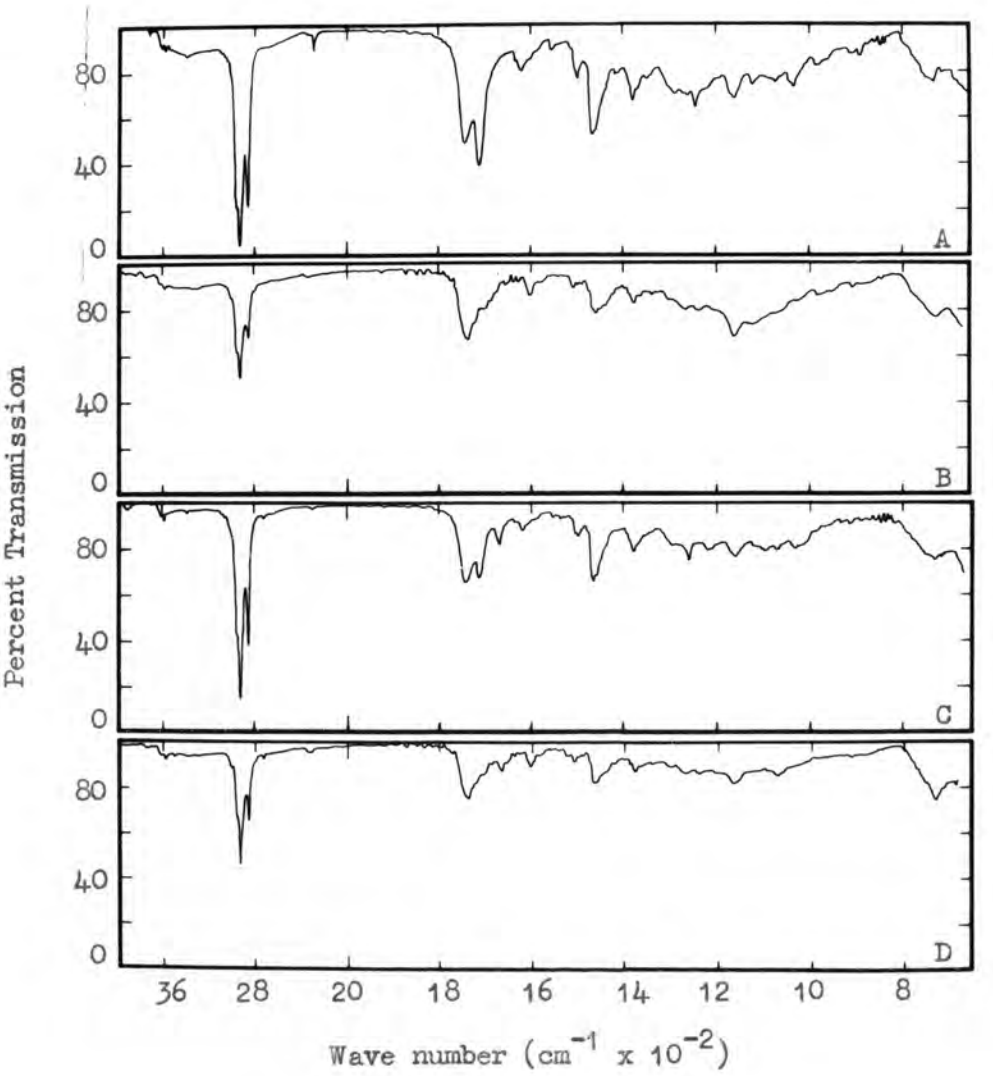
The unusual pigments isolated were designated pigment X (fractions E5,N5; Graph 17), pigment Y (fractions E7.1,N7; Graph 18) and pigment Z (fraction E10; Graph 20). In addition, pigment bands more polar than the preceding compounds showed absorption spectra similar to pigments Z and X (fractions E11,E12; Graph 21) but were not present in sufficient concentration to be characterised further. The visible absorption spectra of pigments X, Y and Z showed similarities to the spectra of pheophytin a, pheophytin b and chlorophyll b respectively, although a number of important differences were apparent (Section 3.311). Apart from the different absorption maxima, pigments X, Y and Z showed higher blue/red absorbance

ratios by approximately 41%, 33% and 58% when compared to the ratios of pheophytin a, pheophytin b and chlorophyll b respectively (Table 4.47).

Diethyl ether solutions of pigments X and Y showed no change in absorption maxima on the addition of hydrochloric acid, although in each case the blue/red absorbance ratio increased slightly (Expt.17.1), more so with pigment X than pigment Y. There was no apparent colour change for either pigment. The addition of acid to an ether solution of pigment Z resulted in a change of colour from green to a yellowish-green, similar to the colour change observed when a solution of chlorophyll b was acidified. The absorption spectrum of acid-treated pigment Z was identical with that of pigment Y and showed principal absorption bands at 427 and 653nm in diethyl ether. The blue/red absorbance ratio increased from 4.44 to 6.95, almost the same as the ratio found for pigment Y. When acid-treated (Mg-free) pigment Z and pigment Y were applied together on a cellulose thin-layer plate, only one band was formed after development, showing the two compounds to have the same polarity under the conditions used for the original separations. These results do not prove, however, that Mg-free pigment Z has the same structure as pigment Y.

The infrared spectra of pigments X and Y were determined (Expt.17.2) on concentrated solutions of the purified pigments and compared to the spectra of pheophytins a and b (Graph 22). Similar absorption patterns were shown throughout most of the wave number range except for a substantial reduction in intensity of the major absorption peak near 1708-1710cm^{-1} in the spectra of pigments X and Y.

Pigments X, Y and Z gave negative reactions in the phase test



GRAPH 22

Infrared spectra of pigments from elevated pH pea puree (solvent C Cl_4).

A	E 2	pheophytin a
B	E 5	pigment X
C	E 4.2	pheophytin b
D	E 7.1	pigment Y

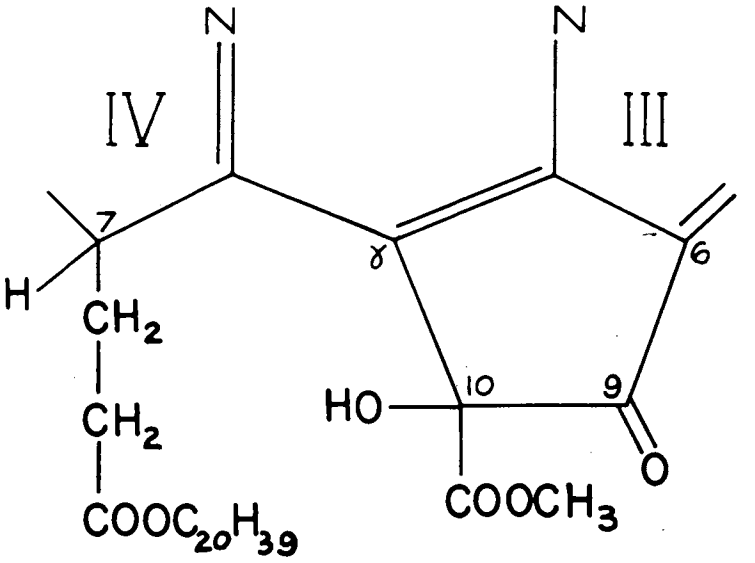
(Expt.17.3) and were not extracted from diethyl ether into dilute potassium hydroxide. The hydrochloric acid numbers (Table 4.48) were lower than those determined for pheophytins a and b.

Before the significance of the above spectral and chemical observations are discussed in relation to possible structures for the unknown pigments, it is of interest to briefly examine recent work on chlorophyll degradation products. In an examination of the effects of various chemical and physical treatments on chlorophylls of leaves and leaf extracts, Bacon and Holden (1967a,b) showed that when boiled leaves were left in aqueous alkaline solutions, or unheated leaves were suspended in aqueous organic solvents, extraction and chromatography of the pigments on cellulose T.L.C. showed the presence of up to six chlorophyll degradation products. Similar compounds were formed from purified pigments incubated in aqueous alkaline or organic solvents, and after chromatography on adsorbents such as silica gel and kieselguhr (Bacon 1966,1967). Three pigments isolated from chlorophyll a were termed "changed" chlorophylls a-1, a-2 and a-3 in order of increasing adsorption on thin layers of cellulose. Similarly, "changed" chlorophylls b-1, b-2 and b-3 were separated from chlorophyll b. Various tests carried out on the "changed" pigments showed that some gave slightly different absorption spectra in various solvents compared to the parent compound. No "changed" pigments were formed when chlorophylls were stored in an atmosphere of nitrogen, all gave negative reactions in the phase test, none were extracted from diethyl ether by potassium hydroxide and all contained the long-chain alcohol phytol. Each "changed" chlorophyll also appeared to form a different Mg-free pigment when acidified.

These results indicated that the "changed" derivatives were oxidised chlorophylls in which the cyclopentanone ring V had been altered. The authors postulated that "changed" chlorophylls a-1 and b-1 were the C-10 hydroxy derivatives (Structure I, Figure 3.2) and that "changed" chlorophylls a-2 and b-2 were possibly the optical isomers at C-10 of a-1 and b-1 respectively (Bacon and Holden 1967a). "Changed" chlorophylls a-3 and b-3 were thought to be of a lactone type containing an oxygen atom between C-9 and C-10 (Bacon 1966) in view of their similarity to pigments described by Holt (1958). The isolation from allomerised chlorophylls of 10-hydroxy chlorophylls a and b and a methoxy-lactone derivative, purpurin 7-lactone - methyl ether - methyl ester (Structure II, Figure 3.2) (Holt 1958), with absorption spectra similar to the compounds described by Bacon and Holden is evidence for the above hypotheses.

The absorption properties of these "changed" pigments are summarised in Table 3.13. When these results are compared to those of pigments X, Y and Z, it is seen that the blue absorption maxima of pigments X, Y and Z are similar to those of Mg-free "changed" a-3, Mg-free "changed" b-3 and "changed" b-3 (or methoxy-lactone chlorophyll b) respectively. The red absorption maxima however, with the exception of pigment Z, are lower than the maxima of the "changed" pigments, even though the shape of the spectra agree fairly well (Bacon 1968). Even allowing for small discrepancies in absorption maxima of the normal pigments (e.g. chlorophylls a and b, pheophytin b) reported in this work (Table 4.43) with those determined by Bacon (1968) (Table 3.13), it would appear that the red absorption maxima of pigments X and Y are too different to allow an

(I)



(II)

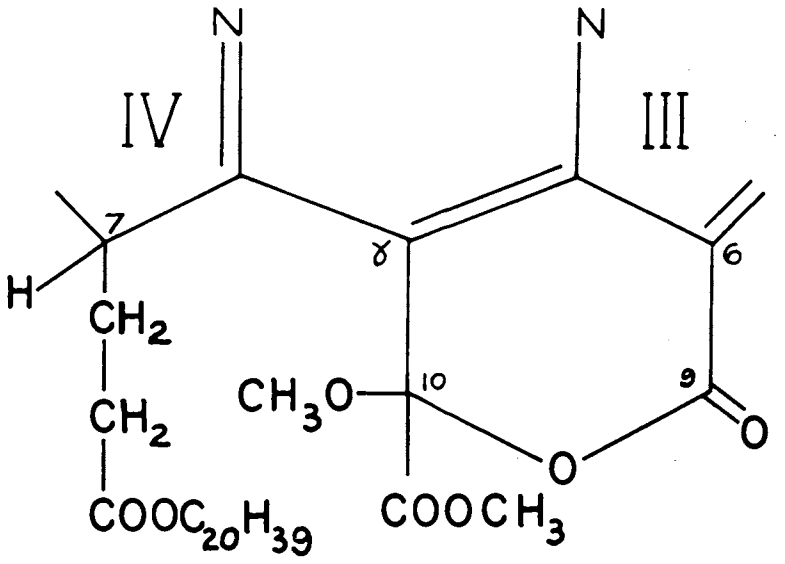


FIGURE 3.2

I 10-hydroxy chlorophyll.

II Purpurin 7-lactone - methyl ether - methyl ester.

identification of these pigments in terms of the "changed" derivatives. Pigments X and Y were, however, phase negative, insoluble in dilute KOH, and appeared to contain phytol as shown by their high acid numbers (Table 4.48) and strong infrared (C-H) absorption between 2800-3000cm^{-1} (Graph 22).

Table 3.13 Absorption Maxima of Chlorophyll Degradation Products

Pigment	Major Absorption Maxima (nm, diethyl ether)	Absorbance Ratio blue/red	Reference
Chlorophyll a	429.5, 661.5	1.28	1
"Changed" Chlorophyll a-1	428 , 661.5	1.28	2
" " a-2	429 , 662	1.26	2
" " a-3	417.5, 653.5	1.90	2
Methoxy-lactone chlorophyll a	416 , 656	1.82	3
Chlorophyll b	453.0, 643.0	2.82	1
"Changed" chlorophyll b-1	451.5, 642	2.98	2
" " b-2	452 , 643	3.21	2
" " b-3	442.5, 631.5	4.60	2
Methoxy-lactone chlorophyll b	442 , 630	4.40	3
Mg-free "changed" a-1	409 , 668.5	2.30	2
" " a-2	408.5, 669.5	2.30	2
" " a-3	403 , 673	3.00	2
Pheophytin b	433 , 655	5.28	2
Mg-free "changed" b-1	432 , 656	5.00	2
" " b-2	432.5, 657.5	3.50	2
" " b-3	426 , 658	4.90	2
Pigment X	401 , 664	2.84-2.93	-
Pigment Y	427 , 653	6.82-6.92	-
Pigment Z	444 , 630	4.44-4.51	-

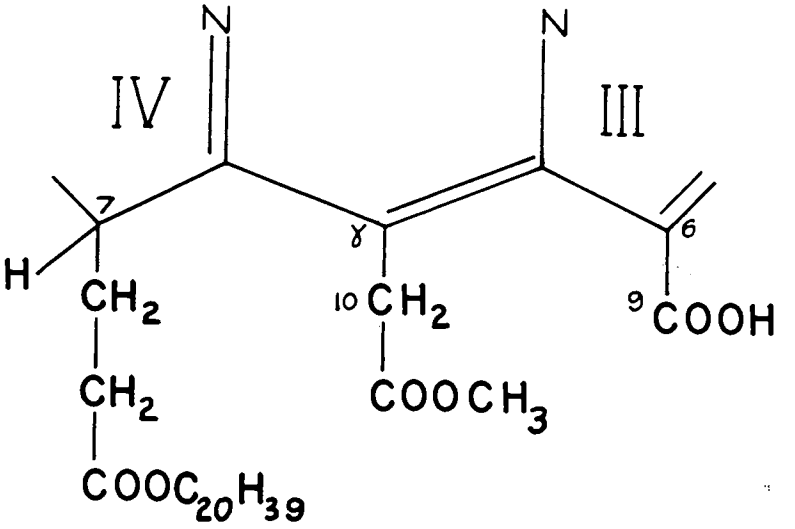
1 Bacon and Holden (1967a)
2 Bacon (1968)
3 Pennington et al. (1967)

The negative phase reaction showed that the cyclopentanone ring had been broken and/or the C-10 hydrogen or carbomethoxy group had been removed (Pennington et al.1964). It is known that under alkaline conditions, the cyclopentanone ring is unstable and can break down with the formation of various derivatives related to chlorin e_6 and other compounds (Seely 1966). Since the highest concentration of unusual pigments was found in elevated pH puree, it appeared that derivatives of this type might more likely correspond in chemical and physical properties with the unusual pigments found.

Briat et al.(1967) examined the effect of various substituents on the absorption spectra of a number of metal-free substituted chlorins, and showed that rupture of the cyclopentanone ring led to absorption changes similar to those observed between pheophytin _a_ and pigment X. For example, methyl pheophorbide _a_ showed principal absorption maxima in dioxane at 411,505,536 and 667nm (blue/red ratio 2.16), while various derivatives without ring V such as chlorin e_6 trimethyl ester (402,500, 530,665nm; ratio 2.94), rhodochlorin dimethyl ester (401,498,527,666nm; ratio 2.45) and isochlorin e_4 dimethyl ester (400,500,527,661nm; ratio 3.04) showed maxima shifted towards the blue and an increased blue/red absorbance ratio. In particular, the "bumps" on the blue side of the Soret peak below 410nm were absent, and the absorption of the secondary maximum at approximately 530nm was greatly decreased.

From these observations, rupture of ring V under alkaline conditions could lead to Structure III (Figure 3.3), a chlorin e_6 derivative, or the isochlorin e_4 derivative Structure IV (Figure 3.3). The presence

(III)



(IV)

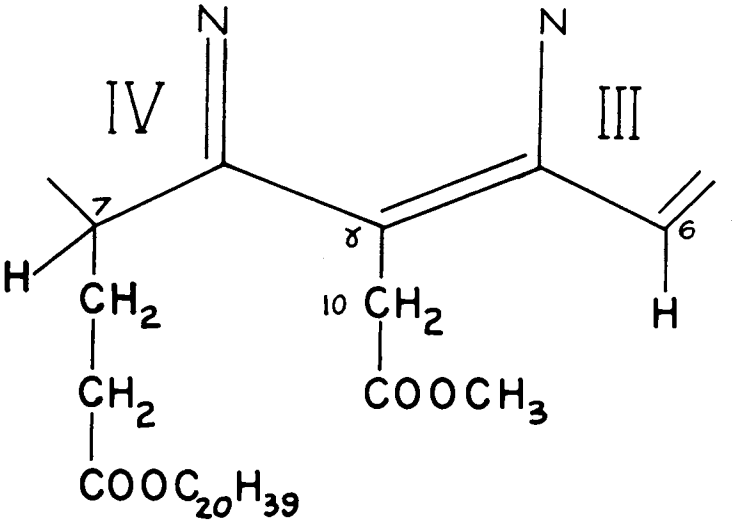


FIGURE 3.3

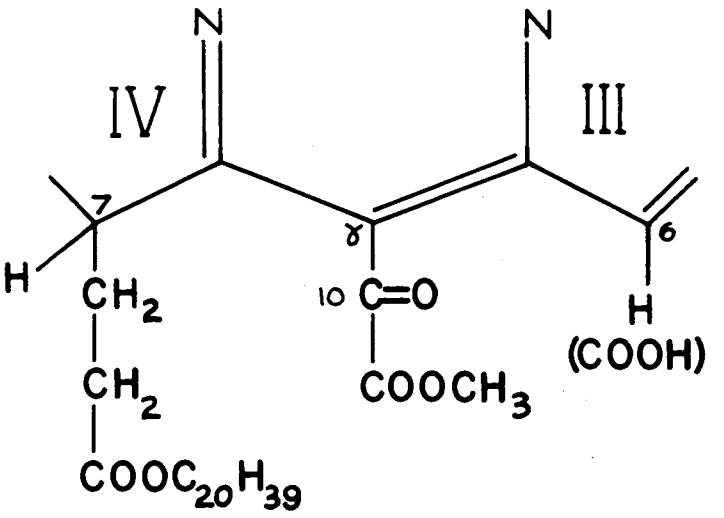
III Chlorin e_6 methyl-phytyl ester.

IV Isochlorin e_4 methyl-phytyl ester.

of a free carboxyl group on C-6, however, would be expected to considerably retard the mobility of a pigment during chromatography, and render it soluble to some extent in dilute alkali. Thus Structure III would appear to be unreasonable in view of the experimental results. Structure IV, which seems more realistic so far as the T.L.C. results are concerned, would require decarboxylation of the C-6 carboxyl group for which elevated temperatures are used in vitro (Fischer and Kellermann 1935). The oxidation of pheophytin under alkaline conditions to produce purpurin 7-derived compounds (Structure V, Figure 3.4) also appears unlikely since these compounds show absorption maxima in the red higher than 680nm (Fischer and Stern 1940).

The retention of a $-CH_2-COOCH_3$ group attached to C-δ is indicated by the infrared results (Graph 22). The spectra of both pigments X and Y showed the presence of an absorption peak near 1740-1745cm^{-1} assigned to vibrations of the C-7 and C-10 ester carbonyls (Katz et al.1966). As these absorption peaks were not decreased in intensity in pigments X and Y compared to pheophytins a and b, this would imply that the two ester carbonyls were retained. The substantial reduction in intensity of the absorption band near 1708-1710cm^{-1}, which has been assigned to vibrations of the C-9 ketone carbonyl group, would appear to indicate that ring V has been opened. However, Holt (1958) showed that in the infrared spectrum of "unstable" chlorin-methyl-phytyl ester (Structure VI, Figure 3.4), the absorption near 1710cm^{-1} occurred only as an inflection on the more prominent ester carbonyl absorption peak near 1740cm^{-1}, similar to the observed spectra of pigments X and Y. A small peak at 3520cm^{-1} indicated the presence of a C-10 hydroxy group in the "unstable" chlorin

(V)



(VI)

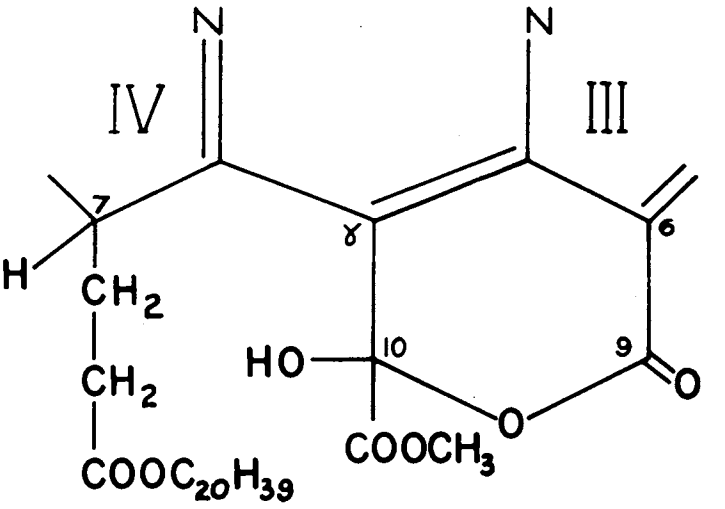


FIGURE 3.4

V Purpurin 7-methyl-phytyl ester.

VI "Unstable" chlorin-methyl-phytyl ester.

compound. Although a peak for a C-10 hydroxyl group was not prominent in the spectra of pigments X and Y, the general absorption near 3400-3600cm^{-1} was increased compared to pheophytin *a* and *b*. It is also known that "unstable" chlorin-methyl-phytyl ester is formed from pheophytin *a* in the presence of alkali and oxygen (Seely 1966) and hence lactone derivatives could be a possible structure for pigments X and Y.

Although pigment Z showed similar spectral and chemical properties to "changed" chlorophyll *b*-3 or methoxy-lactone chlorophyll *b*, (Table 3.13), an identification in terms of these pigments is complicated by the observation that Mg-free pigment Z, identical to pigment Y, was not identical to Mg-free "changed" chlorophyll *b*-3 as previously pointed out. However, it is possible that the Mg phyllin of pigment Y, i.e. pigment Z, has the same spectral properties as the methoxy-lactone compound postulated for "changed" *b*-3 even though the compounds may not be structurally identical. The substitution of -OH or -OCH_3 for the hydrogen at C-10 has been shown to have no effect on the visible spectrum (Holt 1958), and consequently, the assumption that pigment X and Y contain Structure **VI** would still necessarily require that their absorption spectra be similar to those of Mg-free "changed" chlorophylls *a*-3 and *b*-3 respectively which have previously been postulated (Bacon 1966) to contain a lactone configuration at ring V. Since the absorption maxima of the spectra concerned were not identical, it is obvious that both postulates cannot be correct.

The isolation of a slower-moving pigment with a similar spectral pattern to pigment Z (i.e. fraction E11, Graph 21) could nevertheless be explained in terms of a lactone-type structure since two optically

active isomeric forms would be possible. Their close proximity on a T.L.C. plate is in agreement with this hypothesis. The presence of a highly polar pigment (fraction E12, Graph 21) with an identical spectrum to pigment X is difficult to explain. The absence of the phytyl group forming a derivative analogous to pheophorbide a would account for the chromatographic behaviour, but a similar derivative from pigment Y was not found. The pigment was present in very low concentration, and consequently may have been an artifact.

It was of interest to note that no a-series compound corresponding to pigment Z was isolated from the puree extract. The more rapid formation of the b compound, and/or the more rapid conversion of any a compound formed into the Mg-free derivative , i.e. pigment X, would account for this finding, although the first alternative would be contrary to most reactions involving chlorophyll pigments in which the a series have been shown to react faster than the b series.

From these observations, it is apparent that the structures of pigments X, Y and Z cannot be precisely specified from the information available. However, the evidence indicates that they are oxidation products in which (1) a lactone has been formed, or (2) ring V has been opened, or (3) other unknown reactions have occurred. Nevertheless, the isolation of previously unreported chlorophyll degradation products in pH-elevated, processed pea puree shows that similar compounds may be expected in other vegetable products in which the pH has been adjusted for the purpose of pigment retention. The different absorption spectra and chemical properties of these compounds compared to the normal degrad-

ation products, such as pheophytins, indicates the necessity for their chromatographic separation and subsequent estimation, particularly if quantitative pigment estimations are contemplated using spectrophotometric data and appropriate equations.

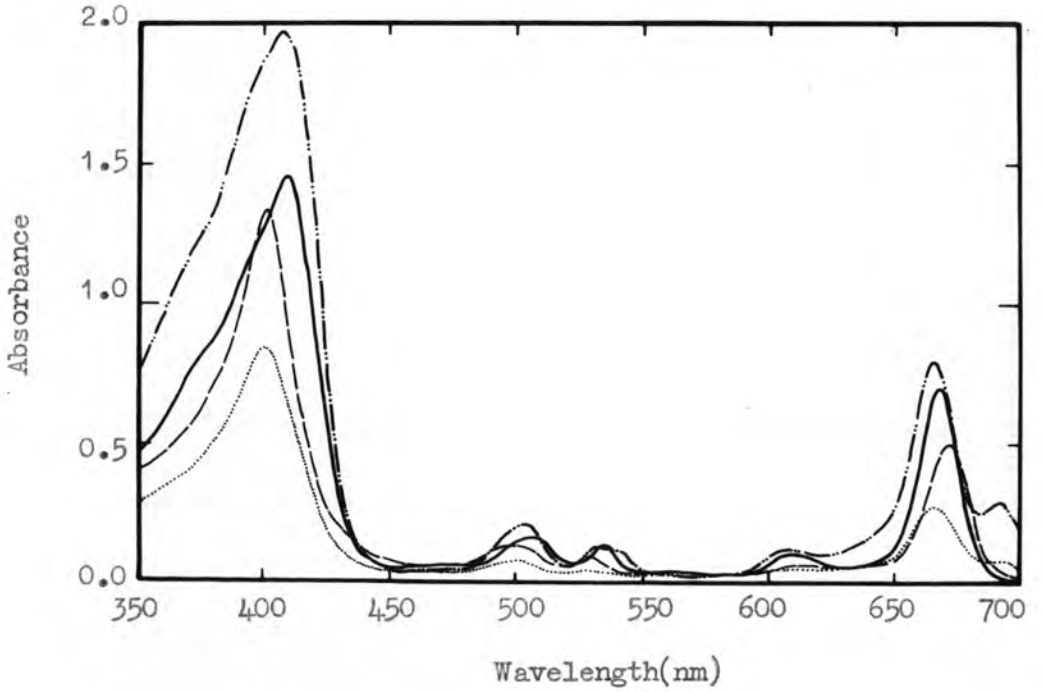
3.4 MODEL SYSTEM STUDIES

It was reported previously that "changed" chlorophyll derivatives were isolated from pure pigments after incubation with alkaline solutions and aqueous organic solvents (Bacon 1966,1967; Bacon and Holden 1967a,b). Because of the overall similarity of these "changed" pigments to the unusual pigments isolated from pea puree in this study, it was of interest to determine whether pigments X, Y or Z were formed under such conditions. Consequently, purified pheophytin a, pheophytin b and chlorophyll b were incubated separately in 50% acetone adjusted to $\frac{M}{100}$ with sodium bicarbonate (Bacon and Holden 1967a,b) and magnesium carbonate (Expt.18). The latter was used to simulate conditions of pH elevation in the processed pea puree.

3.41 Reaction of Pigments with Sodium Bicarbonate

1. Pheophytin a. Chromatography of the pigment incubated for 4 days in the dark at room temperature (Expt.18.11) showed five distinct bands (Table 4.49). The principal grey band B1 (Graph 23) showed similar T.L.C. mobility and absorption maxima in diethyl ether (410,470,506,535,562,609, 667nm) to results previously determined for pheophytin a. The blue/red absorbance ratio (2.08) was unchanged. It was estimated that approximately 60% pheophytin a remained after 4 days.

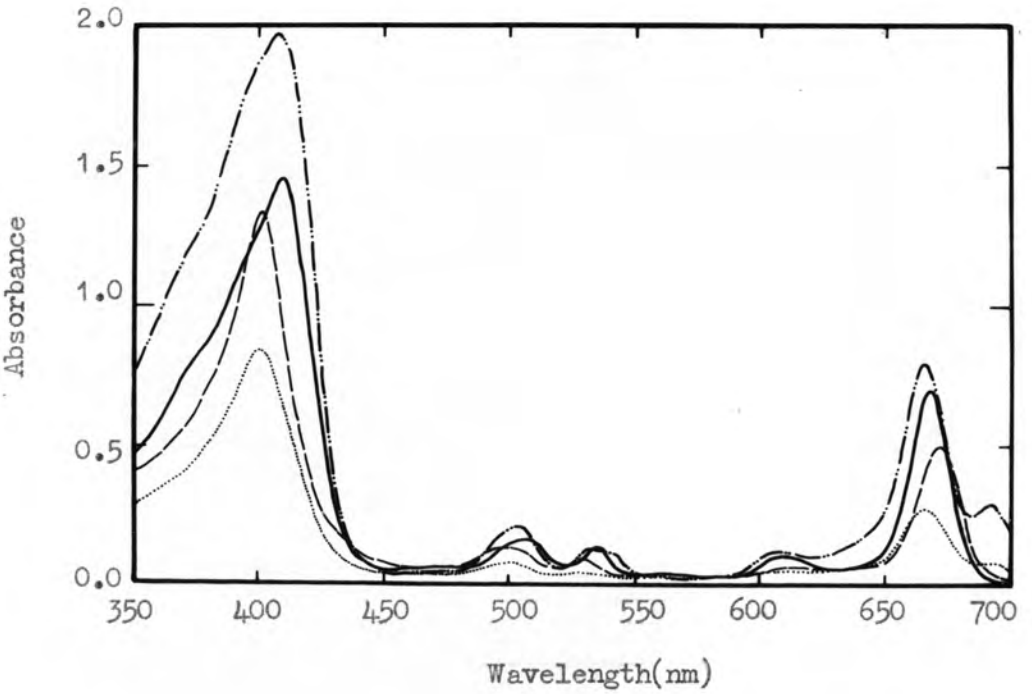
Band B2 (grey, approx. 5%) travelled slightly slower than pheophytin a and showed a very unusual absorption spectrum (Graph 23). Maxima in



GRAPH 23

Spectral curves of pigments in diethyl ether from Pheophytin a incubated 4 days in 50% acetone with sodium bicarbonate.

——— B1 pheophytin a
—·— B2 (40mm)
– – – B3
····· B5 (40mm)



GRAPH 23

Spectral curves of pigments in diethyl ether from Pheophytin a incubated 4 days in 50% acetone with sodium bicarbonate.

——— B1 pheophytin a
—••— B2 (40mm)
– – – B3
..... B5 (40mm)

diethyl ether were 408,504,532,540,608,665 and 692nm, and the blue/red ratio was 2.20 using the 665nm red maximum. Absorption near 690nm was not observed in any chlorophyll pigment previously examined, and it appeared from the absorption at 530-540nm that the solution contained a mixture of two or more pigments. From published reports (Fischer and Stern 1940; Holt 1958) it is possible that the pigment responsible for absorption near 690nm and 540nm was a derivative of purpurin 18 (Structure VII, Figure 3.5) which is known to be produced from pheophytin a or pheophorbide a through the intermediate "unstable" chlorin-ester and/or acid (Structure VI, Figure 3.4) (Seely 1966). Repeated chromatography failed to remove the 692nm absorbing material(s). A compound showing a somewhat similar spectrum with maxima at 404,662.5 and 686nm in diethyl ether, was isolated by Bacon (1968) from a pheophytin a solution incubated 12 days in 80% acetone and $\frac{M}{100}$ in sodium bicarbonate. This pigment ran slower than B2 on a cellulose T.L.C. plate (between C_a and C_b with solvent A) and showed a higher blue/red ratio, but also appeared to be contaminated by some other compound.

Band B3 (grey), approximately 30% of the total pigment concentration, showed absorption maxima at 401,500,530,614 and 671nm in diethyl ether and a blue/red ratio of 2.70. This pigment (Graph 23) appeared very similar to a pigment Bacon (1968) described as having maxima at 400 and 670.5nm, which co-chromatographed with Mg-free "changed" chlorophyll a-3, but which showed a slightly different spectrum (Table 3.13). The spectrum of B3 was different to that of pigment X both in the position of the red maximum, and also the relative heights of the peaks near 530nm and 500nm.

(VII)

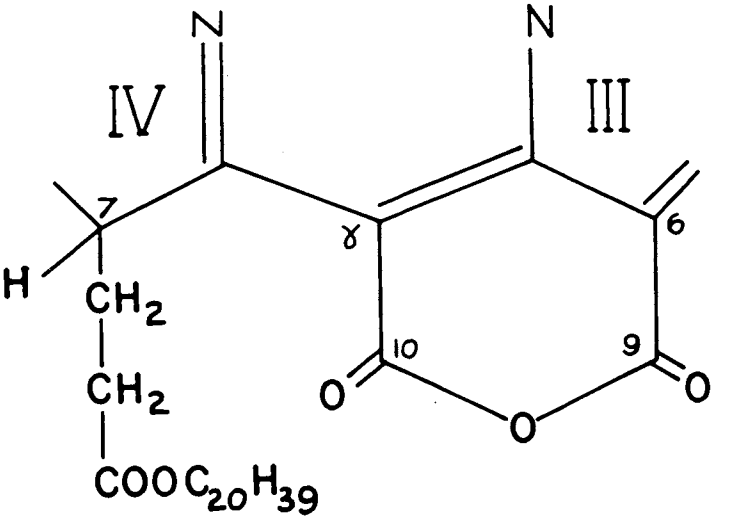


FIGURE 3.5

VII Purpurin 18-phytyl ester.

This result would seem to indicate that pigment X and Mg-free "changed" a-3 are not the same compounds.

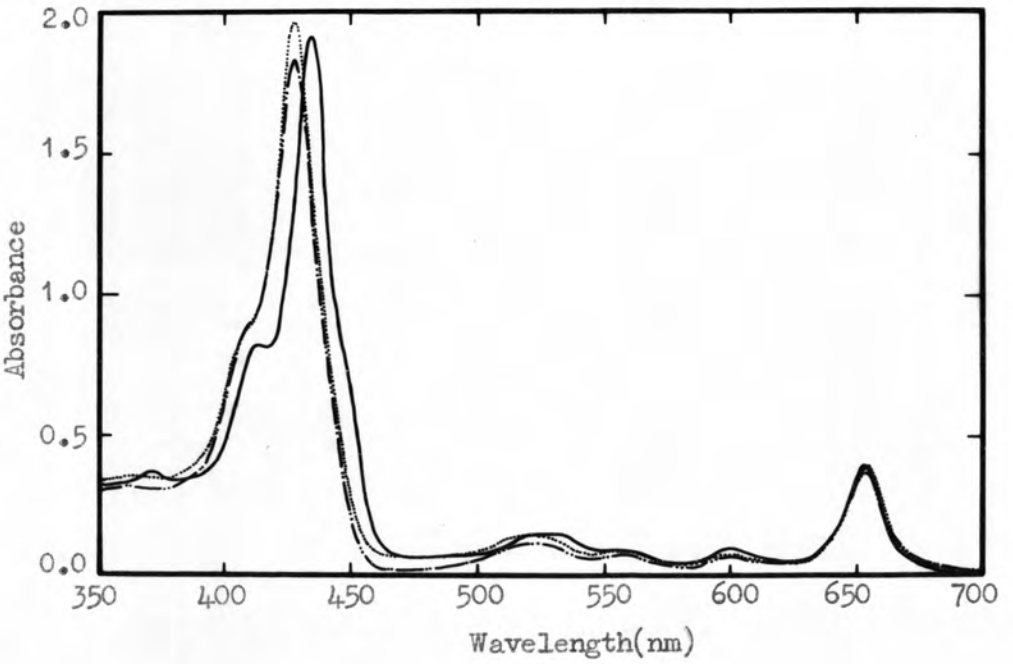
A faint grey band (B4) appeared below B3 but was not present in sufficient concentration for spectral examination.

The last pale grey band B5 (approx. 5%) showed maxima at 400,500, 529 and 665nm in diethyl ether and a blue/red ratio of 3.16 (Graph 23). It appeared very similar to pigment X, but with high absorption near 690nm as found with band B2, and had slightly less mobility than pigment X. In all other respects, it showed a very similar spectrum particularly the absorbance ratio of the secondary maxima at 500-530nm. Pigment B5 was also very similar to a pigment isolated by Bacon (1968) which showed maxima at 400 and 666nm, with a blue/red ratio of 3.19. The absorption pattern near 500-530nm was also similar to pigment X. If pigment B5 is indeed pigment X, this result would also indicate that Mg-free "changed" a-3 is a somewhat different compound to pigment X.

2. Pheophytin b. Purified pheophytin b was also separated into five bands after 4 days incubation (Expt.18.12) but only three (B6,B8,B9) were present in sufficient concentration for rechromatography and spectral analysis. The first yellowish-green band B6 (Table 4.50) showed absorption maxima in diethyl ether at 412,434,524,556,600 and 654nm and was identified as unchanged pheophytin b (Graph 24). This band constituted about 30% of the total pigment concentration.

Band B7 (faint yellow) was not present in sufficient quantity for spectral examination.

Band B8 (yellow-green, approx. 15% total pigment) showed maxima at



GRAPH 24

Spectral curves of pigments in diethyl ether from Pheophytin b incubated 4 days in 50% acetone with sodium bicarbonate.

——— B6 pheophytin b
—•— B8 (40mm)
••••• B9

427,523,557,600 and 653-654nm in diethyl ether with a blue/red ratio of 5.80 (Graph 24). This pigment ran behind pheophytin b, much faster than pigment Y, but its absorption spectrum was very similar to that pigment with the exception of the absorbance ratio.

The major yellow-green band B9 (approx. 55% total pigment) showed mobility slightly slower than chlorophyll a (Table 4.42) and gave absorption maxima in diethyl ether at 427,520,556,599 and 654-655nm, with an absorbance ratio of 5.36 (Graph 24). This pigment appeared similar in chromatographic behaviour and spectral absorption to the major pigment isolated by Bacon (1968) from pheophytin b incubated 12 days in 80% acetone. This latter pigment showed maxima at 426.5 and 655.5nm but an absorbance ratio of 6.1, which was much higher than pigment B9. Bacon's pigment also co-chromatographed with Mg-free "changed" b-3 but showed different absorption maxima (Table 3.13). The minor pigment isolated by Bacon, containing 35% of the total pigment, showed maxima at 427nm and 653nm, identical to pigment Y, but a lower absorbance ratio (6.0 compared to 6.8-6.9) and a higher mobility on cellulose.

The slowest moving band B10 showed similar mobility to pigment Y but was not present in sufficient quantity for spectral identification.

The results of the present work differ somewhat from that of Bacon (1968) but indicate that pigment Y was not produced in any quantity under these incubation conditions. This work also emphasises the great care and caution that must be exercised in attempting to correlate results and conclusions from different laboratories. It was of interest to note that pheophytin b showed a greater conversion to slower-moving

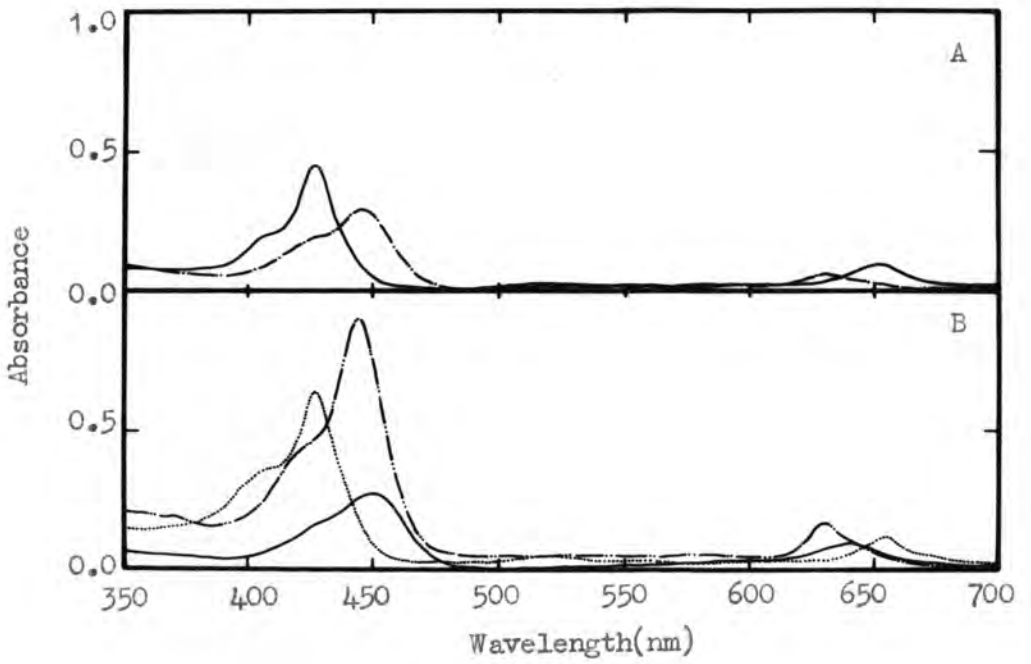
compounds during 4 days incubation (70%) than did pheophytin a (40%). This finding was substantiated by Bacon (1968) and was in agreement with the previous observation that pigment degradation in stored, pH-elevated pea puree occurred to a greater extent with the b series than the a series. However, the conversion of chlorophyll a to pheophytin a occurred at a faster rate than the conversion of chlorophyll b to pheophytin b, in agreement with previous work.

3. Chlorophyll b. Incubated chlorophyll b was converted to four compounds (Expt.16.13, Table 4.51) but only three appeared to be phyllins. The fastest moving band B11 was yellow-green and gave absorption maxima at 427nm and 653nm in diethyl ether (Graph 25a), similar to those previously observed for degradation products of pheophytin b. The absorbance ratio of 6.17 was slightly higher than the ratio for fractions B8 or B9, but was still considerably less than that of pigment Y.

The first green band separated, B12 (approx. 10%) showed absorption maxima (445nm and 630nm, Graph 25a) and mobility similar to pigment Z, but the absorbance ratio of 5.04 was much higher.

Band B13 (green) showed absorption maxima at 451nm and 641nm, with an absorption ratio of 4.00 (Graph 25b). This pigment adsorbed differently to chlorophyll b on cellulose, and was not typical of unreacted chlorophyll b. It was converted to a Mg-free derivative different from pheophytin b.

The major green band B14, (approx.30%), showed similar absorption maxima (444,630nm, Graph 25b) to pigment Z, but the mobility was slower than that pigment. It ran faster than fraction E11, had a spectrum



GRAPH 25

Spectral curves of pigments in diethyl ether from Chlorophyll b incubated 4 days in 50% acetone with sodium bicarbonate.

A. ——— B11 (40mm)
—•—•— B12 (")

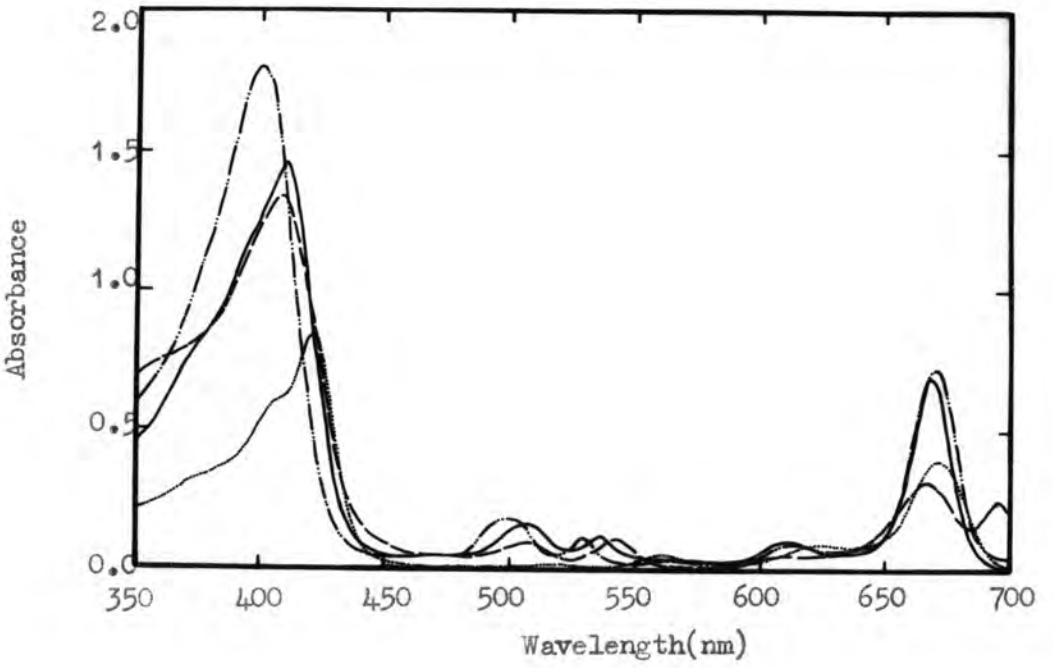
B. ——— B13 (40mm)
—••— B14 (")
••••• B14 acidified (40mm)

similar to pigment Z, and gave an absorbance ratio (5.5) much higher than pigment Z. Acidification of pigment B14 gave a Mg-free derivative with absorption maxima at 427nm and 654-655nm, an absorbance ratio of 5.7, and which co-chromatographed with fraction B11. This Mg-free B14 (Graph 25b) appeared similar to the Mg-free compound (maxima in ether at 426,655nm, ratio 5.5) formed from the major fraction (maxima in ether at 445,635.5nm, ratio 5.1) separated from chlorophyll b incubated 3 days in 80% acetone (Bacon 1968). This latter pigment co-chromatographed with "changed" chlorophyll b-3 but showed slightly different absorption.

The overall observation from this experiment is that pigments X, Y and Z appear to be different from the "changed" chlorophylls and their derivatives, since compounds with properties identical to the unusual X, Y and Z were not found. The pigments separated above showed agreement with some of the results of Bacon (1968) with respect to spectral maxima, absorbance ratio or mobility, and some of the pigments appeared to correlate closely with "changed" b-3, Mg-free "changed" a-3 or Mg-free "changed" b-3.

3.42 Reaction of Pigments with Magnesium Carbonate

1. Pheophytin a. Incubation of pure pheophytin a with magnesium carbonate in 50% acetone (Expt.18.21) produced four pigments separable by cellulose T.L.C. (Table 4.52, Graph 26), three of which were similar to those produced from pheophytin a incubated with sodium bicarbonate. Unreacted pheophytin a was found as the major grey component M1, just ahead of a minor (10%) grey zone M2 also displaying absorption in diethyl ether at 543nm and 695nm (Graph 26) similar to that observed in B2. Band M2, however, appeared to contain a larger proportion of the com-



GRAPH 26

Spectral curves of pigments in diethyl ether from Pheophytin a incubated 4 days in 50% acetone with magnesium carbonate.

——— M1 pheophytin a
– – – M2 (40mm)
– ·· – M3 (")
····· M4 (")

pound, possibly a purpurin 18-derivative, absorbing near 543nm and 695nm as seen by comparing Graphs 23 and 26. The absorbance ratio for M2 was 4.20 for the 666nm red maximum, much higher than the corresponding ratio for B2 (2.20).

Band M3 (30%) was the principal grey degradation product, and showed absorption maxima (400,498,529,614,669nm) and absorbance ratio (2.61, Graph 26) very similar to the corresponding band B3 from the bicarbonate incubation.

The minor, grey, slower pigment M4 (approx. 5%) showed similar mobility but a completely different spectrum to pigment B5 (Graph 26). Its absorption maxima (422,518,561,624 and 671nm) and spectrum agree reasonably well with those tabulated (Holt 1958) for the phyllin of purpurin 18-methyl ester (Structure VII, Figure 3.5). The possible presence of a Mg-containing compound is of interest since the introduction of Mg is considered difficult without the use of Grignard reagents (Seely 1966). The similarity of the spectrum to Mg-purpurin 18-methyl ester would tend to substantiate the tentative identification of a purpurin 18-derivative, undoubtedly the phytyl-ester, as a component of fractions B2 and M2. The phyllin would not be formed, of course, in the sample incubated in sodium bicarbonate.

2. Pheophytin b. The three major fractions isolated from pheophytin b (Expt. 18.22, Table 4.53) were almost identical to the corresponding bands separated after incubation with sodium bicarbonate. Bands M5, M6 and M7 showed similar mobility and almost identical spectral maxima and absorbance ratios to bands B6, B8 and B9 respectively. Band M8 corres-

ponded to B10 in colour and mobility but neither fraction was present in sufficient concentration for spectral analysis. No magnesium complexes were isolated from the incubation mixture.

3. Chlorophyll b. Three breakdown products of chlorophyll b were separated (Expt.18.23) that were different from the original pure chlorophyll b (Table 4.54). Running ahead of unreacted chlorophyll b (band M10) was a yellow-green pigment (M9) with absorption maxima (427,652nm) and absorbance ratio (6.04) similar to band B11. This pigment was also present only as a minor component (10-20%).

Running slower than chlorophyll b was a minor green band M11 similar to band B13 with a high blue/red ratio.

The major green band M12 absorbed similarly to fraction E11, and gave absorption maxima at 445nm and 630nm, with a blue/red ratio of 5.52, almost identical to band B14. It was similar to pigment Z except for the large blue/red ratio.

Comparison of the pigments produced by incubation with sodium bicarbonate and magnesium carbonate in 50% acetone showed only minor differences, such as the derivative from pheophytin a (M4) after incubation with magnesium carbonate. The major pigments were found to be essentially similar to pigments described by Bacon (1968) after incubation with sodium bicarbonate in 80% acetone, but all appeared to be different in some respect (absorption maxima, absorbance ratio or mobility) to pigments X, Y and Z isolated previously from processed pea puree. These results present further evidence that pigments X, Y and Z are not the same as "changed" chlorophylls and their derivatives, but

are apparently compounds similar to those described by Briat et al.(1967).

Although pigments X, Y and Z were of similar colour to the parent compounds and did not give rise to processed materials of unusual colour, nevertheless their presence was indicative of severe chemical alterations to the pigments and also, possibly, to other chemical species present in the plant material and not studied in the present work.

PART II CHLOROPHYLLS, COLOUR AND LIPID OXIDATION IN FROZEN VEGETABLES AND MODEL SYSTEMS

3.5 STORAGE STUDIES ON FROZEN VEGETABLES

3.51 Raw Materials

This section of the work was aimed at investigating the effect of storage time and temperature on chlorophyll and colour changes in frozen peas and beans. Recent work by Walker (1964a,b) has shown that pigment and colour changes in frozen French beans (Phaseolus vulgaris, var. Tendergreen) could be correlated with changes in the oxidation of lipid materials. Implication of the enzyme lipoxygenase in the change of colour in frozen unblanched peas was previously reported by Lee and co-workers (Section 2.222). Recent work on chlorophyll bleaching in legume seed extracts (Holden 1965a) showed that substantial pigment losses were produced in systems containing unsaturated fatty acids and enzymes. These results prompted an investigation of the storage behaviour of peas and beans to examine the relation between pigments, colour and lipid oxidation and to determine the effect of enzymic processes involved.

Samples of fresh peas (Pisum sativum, var. Edgell Freezer) from the same batch as those used in Part I of this work, were blanched or left unblanched, sealed in tinplate containers under an atmosphere of

nitrogen or oxygen, frozen in an air blast at -40°F, and stored at 15°F or -10°F (Expt.19). Peas from the same initial batch were processed and frozen under commercial conditions (Gordon Edgell Pty. Ltd.) and subsequently placed in storage for analysis.

Commercial samples of frozen beans (Phaseolus vulgaris), varieties Tendergreen and Pearlgreen, were obtained from Gordon Edgell Pty. Ltd., Sydney and placed in storage at the two temperatures. Samples of each variety were taken from two growing areas, Bathurst and Brisbane, to determine the influence of growing conditions and environment on storage stability. Samples of frozen materials were removed at storage periods of 0,3,8,13 and 20 months and analysed for (1) chlorophyll pigments, (2) colour, (3) pH, (4) lipid oxidation, (5) chlorophyll bleaching and lipoxygenase activities. Results of enzyme studies are discussed under Model System Experiments (Section 3.6).

3.52 Chlorophyll Pigments

Pigment concentrations were determined by the methods of White et al. (1963), Vernon (1960) and Dietrich (1958) with minor modifications (Expt. 20). Pigment contents for all frozen samples are presented in Tables 4.55 to 4.63.

3.521 Frozen Peas

The changes in pigment content during frozen storage of blanched and unblanched peas were basically in agreement with the results of previous workers (Lindquist et al.1950; Dietrich et al.1955,1957a,b; Boggs et al. 1960). Storage at the higher temperature (15°F) showed substantially greater chlorophyll conversion in all samples than those stored at -10°F.

The effect of blanching, storage time and storage temperature on percent chlorophyll conversion for samples stored in an atmosphere of nitrogen and oxygen are summarised in Table 3.14. Results for the commercial sample are also included.

Table 3.14 Chlorophyll Conversion During Storage of Blanched and Unblanched Frozen Peas

Sample	Storage Temperature (°F)	Percent Chlorophyll Conversion				
		Storage Time (Months)				
		0	3	8	13	20
Blanched, N_2	15	4.0	9.6	13.0	18.2	22.3
Blanched, O_2	"	4.0	8.3	13.7	20.0	25.8
Blanched, Commercial	"	6.2	9.3	18.1	23.4	27.1
Unblanched, N_2*	"	6.4	26.0	48.9	69.0	83.1
Unblanched, O_2*	"	6.4	31.3	56.8	70.4	87.0
Blanched, N_2	-10	4.0	3.2	5.1	6.0	8.3
Blanched, O_2	"	4.0	6.2	4.6	4.2	7.8
Blanched, Commercial	"	6.2	3.5	5.8	6.7	7.2
Unblanched, N_2*	"	6.4	7.0	10.3	8.1	13.9
Unblanched, O_2*	"	6.4	7.3	14.4	12.1	18.8

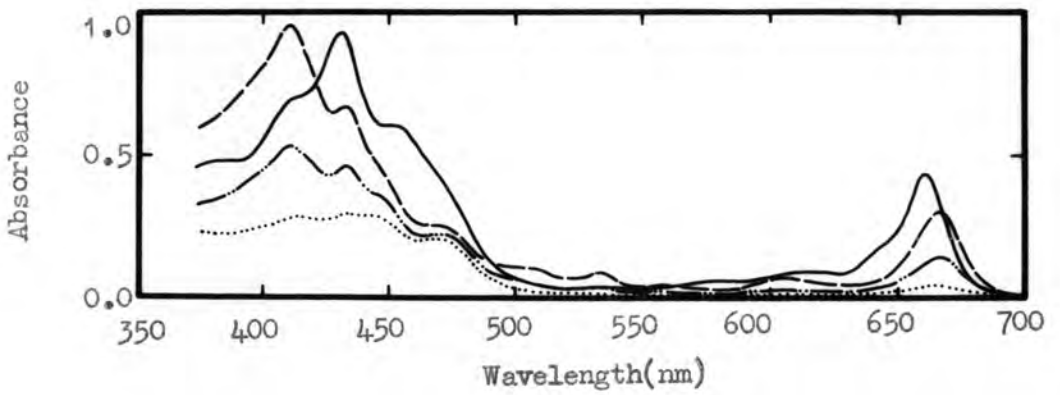
* Conversion of chlorophylls + chlorophyllides to pheophytins + pheophorbides.

The results showed clearly that blanching had a considerable effect on the retention of chlorophylls in frozen peas, particularly at elevated storage temperatures. Blanched peas stored at -10°F showed a 4% increase in percent chlorophyll conversion in 20 months, compared to increases of 7.5% and 12.2% for unblanched peas stored at -10°F under nitrogen and oxygen respectively. At 15°F, blanched peas under nitrogen and oxygen showed increases in chlorophyll conversion of 18.3% and 21.8%, while the corresponding figures for unblanched peas were 76.7% and 80.6% respectively. One of the most important purposes of blanching in frozen

vegetables is the inactivation of enzymes, and in this regard operations are designed to destroy the enzyme peroxidase, one of the most heat resistant enzymes in plant materials. Unblanched vegetables have been shown to retain considerable enzymic activity during prolonged frozen storage, and the activity of lipases, which result in the liberation of free fatty acids from fat glycerides, are one of the principal reasons for the loss of chlorophylls during storage.

The commercially frozen peas showed slightly higher initial conversion than samples prepared in the laboratory, presumably due to their pre-storage treatment, and after 20 months storage at 15°F also showed greater conversion of chlorophylls. Blanched peas stored under nitrogen at 15°F showed less conversion than samples stored under oxygen. The presence of oxygen also increased chlorophyll conversion at both temperatures in the unblanched samples, although the largest difference was observed at the lower temperature (-10°F).

In all cases, it was observed that chlorophyll a degraded to pheophytin a at a faster rate than did chlorophyll b to pheophytin b. No phytol-free pigments were found in any blanched samples, and this was subsequently substantiated by pigment separation using thin-layer chromatography (Expt.35). A considerable quantity of phytol-free pigments, i.e. chlorophyllides and pheophorbides, were found in all unblanched samples, even at the low storage temperatures (Tables 4.58,4.59). Graph 27 shows selected spectra of extracts from unblanched peas stored under oxygen, and indicates that the formation of phytol-free pigments increased with the temperature and time of storage. The changes in con-



GRAPH 27

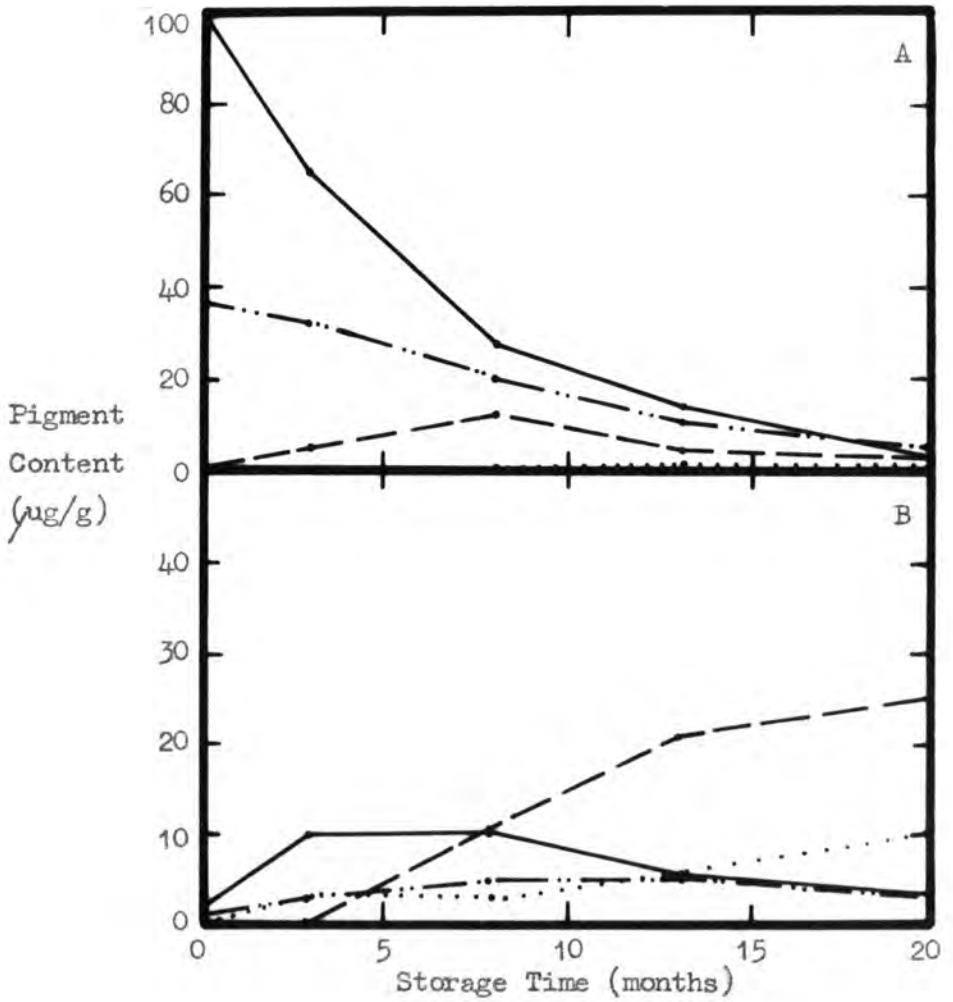
Spectral curves of gross pigment extracts from frozen unblanched peas.

——— Stored 8 months under oxygen at -10°F, ether solution 1.
– – – " " " " " " " " 15°F, " " 4.
–•–•– " " " " " " " " 20 " " " " " " " " " " " " 4.
••••• " 4.

centration of the individual pigments are shown in Graph 28.

Unblanched, brined cucumbers have been shown to contain phytol-free pigments (White et al.1963), but the presence of these derivatives in unblanched frozen peas has not previously been reported. The enzyme chlorophyllase (Section 2.2131) is widely distributed in flowering plants, and its activity is concentrated in the chloroplast fraction. Although it is now believed that the final step in the biosynthesis of chlorophyll a is the esterification with phytol of the propionic acid group at C-7 of chlorophyllide a (Bogorad 1965,1966), the activity of this enzyme in the degradation of chlorophyll is not well understood (Holden 1967b).

It is of interest to note that the formation of phytol-free pigments increased with increasing storage time (Table 3.15), more so at the higher storage temperature. Possibly the liberation of active enzyme was induced by cellular damage caused by an increased proportion of ice present at the low temperatures, but evidence to support this supposition was not collected. Attempts to show the presence of high concentrations of chlorophyllase were inconclusive. The identification of phytol-free pigments was substantiated by thin-layer chromatography of acidified pigment extracts from stored unblanched frozen peas (Expt.35.1), and it was found that the absorption maxima of fractions F6 and F7 (Table 4.92) were almost identical to previous results for pheophorbide a and pheophorbide b (Table 4.03). Pigments F6 and F7 showed much greater polarity on cellulose than their phytylated derivatives F2 and F4 respectively, in agreement with the presence of a free carboxyl group at C-7. The phytol-free pigments also showed lower hydrochloric acid numbers similar



GRAPH 28

Changes in Chlorophyll pigments in frozen unblanched peas stored under nitrogen for 20 months at 15°F.

A. ——— chlorophyll a
– – – chlorophyllide a
–·· – chlorophyll b
····· chlorophyllide b

B. ——— pheophytin a
– – – pheophorbide a
–· ·– pheophytin b
····· pheophorbide b

to those observed previously and were extractable into dilute alkali from diethyl ether. The chromatography of an acidified extract from frozen blanched peas stored for 20 months at -10°F (Expt.35.2) showed the presence of pheophytin a, pheophytin b and four carotenoid pigments (Table 4.93), but no phytol-free derivatives.

Table 3.15 Formation of Phytol-Free Pigments During Storage of Frozen Unblanched Peas

Sample	Storage Temperature (°F)	Percent Conversion C Cd[1] Storage Time (Months) 0	3	8	13	20	Percent Conversion Py Po[2] Storage Time (Months) 0	3	8	13	20
Unblanched, N_2	15	0.0	7.2	21.5	22.9	37.6	0.0	26.5	48.0	71.0	86.6
Unblanched, O_2	"	0.0	0.0	10.4	21.7	65.9	0.0	23.8	59.9	67.7	81.7
Unblanched, N_2	-10	0.0	0.0	0.0	3.7	4.7	0.0	0.0	13.3	26.4	28.9
Unblanched, O_2	"	0.0	0.0	0.0	3.3	5.8	0.0	0.0	4.8	17.1	24.8

1 - Conversion of chlorophyll (a + b) to chlorophyllide (a + b)

2 - Conversion of pheophytin (a + b) to pheophorbide (a + b)

Apart from the pigment conversions discussed above, frozen unblanched peas also showed a loss of total pigment during storage, both at 15°F and -10°F (Table 3.16). The greatest loss, in samples stored for 20 months at 15°F under oxygen amounted to 52.0µg/g or 35.6%, while only 6.0% pigment was destroyed under nitrogen at -10°F. The samples stored under nitrogen at 15°F, however, showed 29.6% pigment destruction and indicated that if oxidative reactions were involved, then sufficient oxygen tension existed in the tissues to promote such changes despite the repeated nitrogen flushing that had been applied. Results of other analyses on frozen unblanched peas suggest that oxidative enzymic reactions were probably responsible for the pigment losses. These results are discussed

in greater detail in Sections 3.54 and 3.63.

Table 3.16 Total Pigment Content of Frozen Unblanched Peas

Sample	Storage Temperature (°F)	Total Pigment Content (μg/g) Storage Time (Months) 0	3	8	13	20
Unblanched, N_2	15	145.7	143.8	121.8	112.5	102.5
Unblanched, O_2	"	145.7	135.9	111.1	106.0	93.7
Unblanched, N_2	-10	145.7	143.2	137.8	147.6	136.9
Unblanched, O_2	"	145.7	147.6	143.7	135.0	124.3

3.522 Frozen Beans

Results of pigment determination on frozen beans stored for 0,3,8, 13 and 20 months are presented in Tables 4.60 to 4.63 inclusive (Expt. 20.2). Differences between the four samples (2 varieties, 2 growing areas) can be seen more clearly in Table 3.17. It will be noticed that the two varieties, Tendergreen (TG) and Pearlgreen (PG), grown and processed in Brisbane (BB) showed slightly higher initial chlorophyll conversion than those processed at Bathurst (BH), and after storage for 20 months at both 15°F and -10°F this trend was still apparent. One of the reasons for the greater initial conversion in the Brisbane samples was their storage at 0°F for six weeks before despatch to Sydney. Nevertheless, it is of interest to note that the Brisbane samples were also given a more severe blanching treatment ($3\frac{1}{4}$ minutes in steam at 210°F) than the Bathurst samples (2 minutes in steam at 205°F) which, as Walker (1964b) has pointed out, can result in increased pigment destruction during storage from mechanisms involving the induced oxidation of lipids.

Table 3.17 Chlorophyll Conversion During Storage of Frozen Beans

Sample	Storage Temperature (°F)	Percent Chlorophyll Conversion Storage Time (Months) 0	3	8	13	20
TG, BH	15	13.6	35.3	57.5	72.0	82.7
TG, BB	"	15.1	39.0	64.8	79.2	84.5
PG, BH	"	12.7	33.6	58.7	74.0	82.8
PG, BB	"	14.2	39.0	56.3	67.7	88.2
TG, BH	-10	13.6	18.2	14.1	15.2	16.0
TG, BB	"	15.1	14.6	16.9	20.0	18.9
PG, BH	"	12.7	16.3	14.7	15.6	15.4
PG, BB	"	14.2	15.1	13.7	18.3	22.8

Changes in total pigment content (Table 3.18) were not consistent with changes in chlorophyll conversion. No significant differences were observed during storage at -10°F, although Pearlgreen-Brisbane beans showed a larger change than did the other samples. At 15°F storage, small decreases in total pigment were found with the Pearlgreen variety at both growing areas, more so with the Bathurst sample (8.8%) than the Brisbane sample (5.9%). No losses were observed in the Tendergreen variety, contrary to the findings of Walker (1964a).

Table 3.18 Total Pigment Content of Frozen Beans

Sample	Storage Temperature (°F)	Total Pigment Content (ug/g) Storage Time (Months) 0	3	8	13	20
TG, BH	15	101.8	104.5	102.7	98.9	99.6
TG, BB	"	79.7	80.1	77.0	81.6	80.8
PG, BH	"	67.0	66.7	65.8	62.0	60.1
PG, BB	"	75.6	74.9	76.1	74.4	71.1
TG, BH	-10	101.8	101.8	103.8	97.3	101.4
TG, BB	"	79.7	79.0	80.6	83.4	81.8
PG, BH	"	67.0	68.1	66.8	67.0	66.3
PG, BB	"	75.6	74.0	75.3	76.5	73.8

Another point of interest was the considerable difference in initial total pigment content, particularly between the same variety, Tendergreen, grown at different locations, i.e. Bathurst (101.8µg/g) and Brisbane (79.7µg/g). It was also interesting to note that the lowest pigment content (67.0µg/g) was in the Pearlgreen-Bathurst samples which also showed the greatest loss in total pigment during storage at 15°F.

In agreement with previous results on peas, chlorophyll a conversion was more rapid than that of chlorophyll b, with 95% chlorophyll a lost during 20 months storage at 15°F, but less than 70% chlorophyll b destroyed under the same conditions. There was, however, a considerable difference between chlorophyll conversion in blanched peas compared to that in beans (Tables 3.14,3.17), and the much higher pigment conversion in beans was in agreement with their lower pH (Section 3.55).

3.53 Colour Measurements

3.531 Frozen Peas

Hunterlab measurements were determined on thawed, pureed material since preliminary experiments had shown that samples in this form gave similar results to whole material but with less variation between replicate determinations. The Hunterlab indices (Expt.21.2, Tables 4.64 to 4.68) showed similar trends to total chlorophyll or percent chlorophyll conversion discussed previously. It is obvious from the tabulated results that (1) blanched peas showed much less colour change than unblanched peas; (2) samples stored under nitrogen showed less colour change than samples stored under oxygen, whether blanched or unblanched.

The greatest numerical change occurred in the '-a' value, which

varied from -19.5 to -4.1, although the 'L' and 'b' values also changed but in somewhat different directions for blanched and unblanched samples. Blanched peas, whether stored under nitrogen or oxygen or commercially prepared, showed decreases in 'L' and 'b' and increases in 'a' values at both storage temperatures with greater changes occurring at the higher temperature. Changes in the 'a' value were generally greater than changes in the 'L' value, while changes in 'b' were relatively small. For unblanched peas, the 'a' value increased markedly and the 'b' value decreased substantially compared to the change in blanched peas. This loss of "yellowness" may have been due to a loss of carotenoid pigments coupled to enzymic lipid oxidation, but was not studied quantitatively. Concurrent with this decrease in 'b' value there was a significant increase in the lightness dimension 'L' at 15°F. This is in agreement with the previous finding that total pigment content decreased in these samples. The loss of green intensity, together with a decrease in the yellow components, produced an overall lighter, less coloured surface.

Values for each of the derived Hunterlab indices $^{-a}/_{b}$, $^{-a}/_{L}$ and $(a^2+b^2)^{\frac{1}{2}}$ decreased with increasing storage time in agreement with the pigment data. It was of interest, therefore, to determine the correlation between pigment results and the derived Hunterlab colour indices. Correlation coefficients are shown in Table 3.19. Correlations between pigment conversion to the Hunterlab indices $^{-a}/_{b}$ and $^{-a}/_{L}$ were very highly significant. The index $(a^2+b^2)^{\frac{1}{2}}$ showed a less significant correlation for blanched peas ($r = -0.882$) than for unblanched peas ($r = -0.936$), in agreement with the observed development of "less intense" green colours in unblanched peas as a result of chlorophyll, and/or carotenoid destruction.

Table 3.19 Correlation Between Chlorophyll Conversion and Objective Colour Indices for Stored Frozen Peas

Sample	Relationship	Correlation Coefficient "r"
Blanched Peas	Percent Chlorophyll Conversion vs Index $^{-a}/_{b}$	-0.969
" "	Percent Chlorophyll Conversion vs Index $^{-a}/_{L}$	-0.953
" "	Percent Chlorophyll Conversion vs Index $(a^2+b^2)^{\frac{1}{2}}$	-0.882
" "	Index $^{-a}/_{b}$ vs Index $^{-a}/_{L}$	0.984
Unblanched Peas	Percent Chlorophyll Conversion vs Index $^{-a}/_{b}$	-0.975
" "	Percent Chlorophyll Conversion vs Index $^{-a}/_{L}$	-0.975
" "	Percent Chlorophyll Conversion vs Index $(a^2+b^2)^{\frac{1}{2}}$	-0.936
" "	Index $^{-a}/_{b}$ vs Index $^{-a}/_{L}$	0.993

Hunterlab values and particularly the 'a' value and derived colour indices were higher (more negative 'a' values) for blanched peas than for unblanched peas. The apparent increased greenness as a result of blanching was in agreement with the observed colour change to a more intense green as a result of expulsion of air from the tissues. This fact was particularly noticeable when preparing purees for colour measurements; unblanched peas contained considerable air and pureed samples had to be deaerated thoroughly so that erroneous 'L' values were not recorded.

3.532 Frozen Beans

Before storage trials were commenced samples of frozen beans were visually ranked in order of "desirable green bean colour" by a panel of four judges. The samples were ranked whole and as a puree, and Hunterlab values determined. Results for the pureed samples are presented in Table 3.20. Visual ranking in order of desirable green colour also ranked the samples in order of decreasing total chlorophyll content. However, no colour index showed a similar trend. For example, Pearlgreen-

Bathurst samples were visually ranked 3, but showed the highest $^{-a}/L$ and $(a^2+b^2)^{\frac{1}{2}}$ indices and the lowest $^{-a}/b$ index. 'L', 'a' and 'b' values for this sample were all higher (more negative 'a' values) than for the other 3 samples. Pearlgreen-Brisbane, on the other hand, showed the lowest percent conversion and total chlorophyll, but the highest $^{-a}/b$ index.

Table 3.20 Visual Rank, Pigment Content and Objective Colour Indices for Frozen Beans

Sample	Mean Visual Rank	Percent Chlorophyll Conversion	Total Chlorophyll Content (µg/g)	L	a	b	Ratio $^{-a}/b$	Ratio $^{-a}/L$	Index $(a^2+b^2)^{\frac{1}{2}}$
TG-BH	1	13.6	88.0	37.9	-13.5	18.8	0.718	0.356	23.15
TG-BB	2	15.1	67.7	37.3	-13.7	19.1	0.717	0.367	23.50
PG-BH	3	14.2	64.9	38.9	-14.7	20.7	0.710	0.378	25.40
PG-BB	4	12.7	58.5	37.4	-14.0	19.2	0.729	0.374	23.76

It is obvious from these observations that colour indices were not useful for specifying the colour as subjectively assessed, even though the pigment data suggested large colour differences. Changes in colour indices with storage, however, (Tables 4.69 to 4.72) did show trends similar to the changes observed in pigment content and chlorophyll conversion. When correlation coefficients were calculated relating percent chlorophyll conversion to the Hunterlab indices $^{-a}/b$, $^{-a}/L$ and $(a^2+b^2)^{\frac{1}{2}}$, significant results were obtained (Table 3.21). The correlation between chlorophyll conversion and the index $(a^2+b^2)^{\frac{1}{2}}$ was statistically much lower than correlations involving the hue ratio $^{-a}/b$ or the ratio $^{-a}/L$, in agreement with the work of Dietrich et al.(1957b), who showed that for frozen beans, changes in hue were considerably more meaningful than changes in saturation in expressing colour changes during storage.

Table 3.21 Correlation Between Chlorophyll Conversion and Objective Indices for Stored Frozen Beans

Relationship	Correlation Coefficient "r"
Percent Chlorophyll Conversion vs Ratio $^{-a}/_{b}$	-0.950
Percent Chlorophyll Conversion vs Ratio $^{-a}/_{L}$	-0.959
Percent Chlorophyll Conversion vs Index $(a^2+b^2)^{\frac{1}{2}}$	-0.835
Ratio $^{-a}/_{b}$ vs Ratio $^{-a}/_{L}$	0.983

It is apparent that objective measurements of colour on stored frozen peas and beans give a reasonably good indication of pigment changes in terms of percent chlorophyll conversion to pheophytin. Use of objective colour data for estimation of actual pigment content, however, would appear to lead to erroneous conclusions when only chlorophyll pigments are considered, although the small sample size studied here would make definite conclusions difficult. The close relationship between pigment breakdown and overall quality of frozen materials has previously been reported by many workers and it would appear from this study that objective colour measurements could give reliable indications of quality deterioration providing standardised procedures were adopted.

3.54 Lipid Oxidation

3.541 Methods for Determining Lipid Oxidation

Methods for the measurement of oxidative deterioration of lipid materials have been examined in detail for some considerable time. The importance of lipid oxidation and associated reactions in the storage behaviour of fresh and processed food materials has prompted the search for accurate and reliable methods of measuring fat oxidation which can

be correlated with subjective evaluations of rancid odours and flavours. In general, methods employed have been concerned either with the estimation of conjugated hydroperoxides, the primary product of oxidation of unsaturated fatty acids (Lundberg 1961), or estimation of degradation products of hydroperoxides. The former include the iodometric and ferric thiocyanate methods while the latter include the 2-thiobarbituric acid method and the estimation of total and volatile carbonylic compounds.

The 2-thiobarbituric acid (TBA) test has been widely used for measuring oxidative changes in foods containing unsaturated fatty acids. Kohn and Liversedge (1944) reported that a red colour was formed when tissue suspensions or slices were incubated aerobically with TBA, and suggested that the TBA-reactive material was a carbonyl compound. Later results by other workers implicated the three-carbon compound malonaldehyde ($OHC-CH_2-CHO$) as responsible for the red TBA pigment (Patton et al. 1951). Sinnhuber et al.(1958) isolated a red pigment from rancid salmon oil and showed it to be a condensation product of 1 molecule of malonaldehyde and 2 molecules of TBA. This pigment was identical to that formed between TBA and the acid hydrolysis product of 1,1,3,3,-tetraethoxypropane (Sinnhuber and Yu 1958).

A number of empirical procedures using the TBA reaction as a measure of oxidative rancidity have appeared in the literature, and the test has been used to measure lipid oxidation in various dairy products (Sidwell et al.1955), frozen pork (Turner et al.1954), fats and oils (Pohle et al. 1964), meat products (Tarladgis et al.1960), fishery products (Yu and Sinnhuber 1967) and frozen peas (Rhee and Watts 1966a). Despite the

widespread use of the TBA method, there has been doubt as to the actual presence of malonaldehyde in extracts from autoxidised unsaturated fatty acids (Saslaw et al.1963,1966) although reports on autoxidised methyl linolenate and other highly unsaturated fatty acids have shown the presence of a major component unequivocally identified as malonaldehyde (Kwon and Olcott 1966).

Dahle et al.(1962) in a study of the TBA reaction on autoxidised polyunsaturated fatty acid esters, showed that esters with 3 or more double bonds gave TBA colours, but that autoxidised oleic and linoleic esters gave no reaction in pure samples during early stages of oxidation. Peroxide values (iodine titration), diene conjugation (UV absorption) and oxygen absorption, however, increased with increasing unsaturation of the ester. These results showed that the TBA colour, developed from autoxidised lipids of tissue samples, varied with the profile of polyunsaturated fatty acids present, and that the TBA reaction had only qualitative and comparative significance.

Tarladgis et al.(1960) described a method for the quantitative determination of malonaldehyde in rancid foods in which the food slurry was distilled at low pH, TBA solution was added to an aliquot of the aqueous distillate and the lipid oxidation determined from the absorbance of the red solution by reference to a calibration curve of malonaldehyde derived from 1,1,3,3,-tetraethoxypropane. Previous workers had shown that acid and heat were required to extract malonaldehyde from rancid tissues and for the condensation of malonaldehyde with TBA. Later results of Tarladgis et al.(1962,1964) indicated that acid-heat treatments resulted

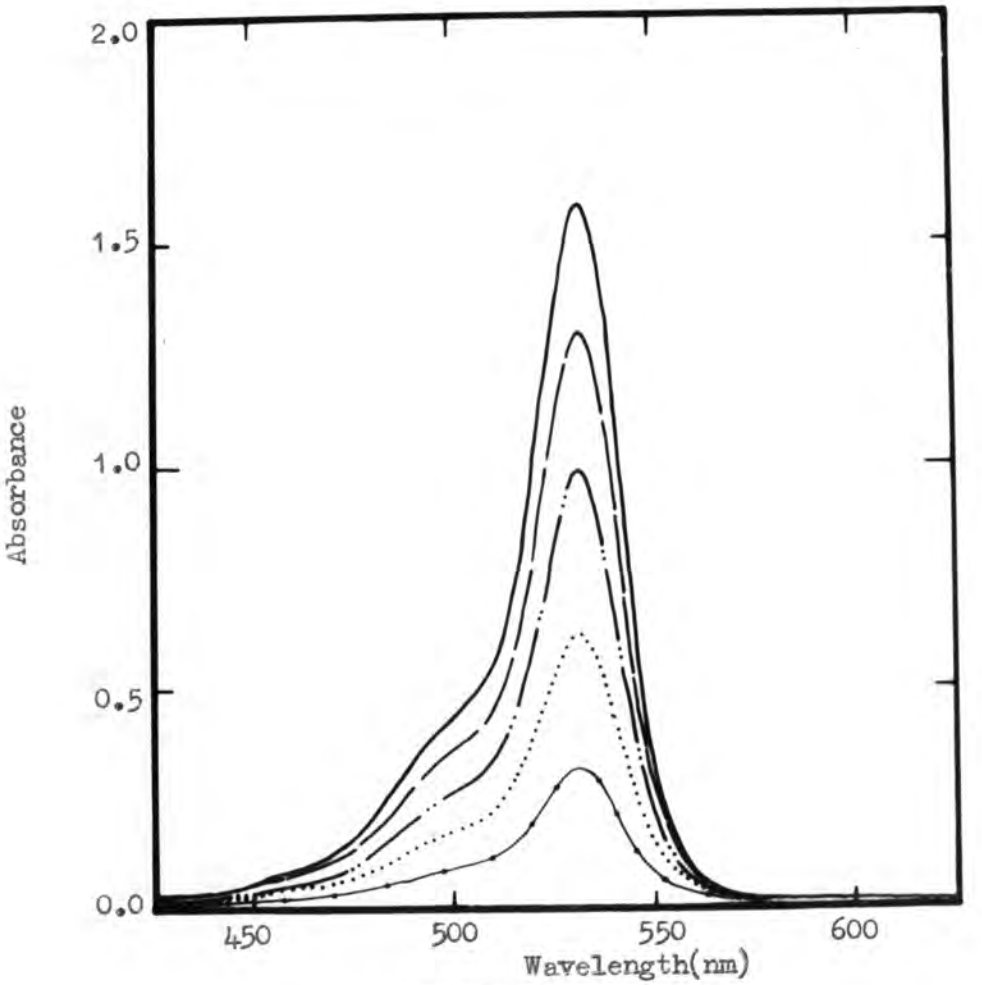
in various side reactions and breakdown of the TBA reagent, and they advocated the use of a modified method in which acid-heat treatment was minimised. For samples in which clear water extracts could not be obtained for TBA tests, however, the distillation procedure was necessary. Some of these side reactions were later shown by Yu and Sinnhuber (1964) to be caused by the use of impure reagents, and they stressed the importance of this aspect in the interpretation of TBA results.

Although some reports showed that the TBA reaction was somewhat limited in its application and must be carefully used to obtain meaningful results (Kwon et al.1965), it has nevertheless showed good correlation with other fat oxidation indices and with subjective flavour and odour assessments on various foods, particularly in the early stages of oxidation. At the time of the commencement of the present work, the TBA test had not previously been used for the estimation of lipid oxidation in frozen peas and beans. However, Rhee and Watts (1966b) recently examined the oxidation of lipids in frozen and stored raw, blanched and cooked Blackeye peas (Vigna sinensis) by a modification of the TBA distillation method, and showed that the TBA number (mg malonaldehyde/1000g sample) of raw peas increased during storage at 14°F, while blanched and cooked peas showed no change. Their results indicated, however, that lipid oxidation was not the principal cause of off-flavour in frozen unblanched peas.

The TBA test was used as a measure of lipid oxidation in frozen peas and beans in this study as a comparison with results for hydroperoxide content as measured by the ferric thiocyanate method. The presence of

active enzymes in unblanched peas, in particular lipoxygenase, has been demonstrated to cause peroxidation of lipids (Lee and Wagenknecht 1958) and peas and beans are known to contain large quantities of polyunsaturated fatty acids (Wagenknecht 1957,1963; Lee and Mattick 1961; Forman and Pokorny 1966). Consequently, a measurement of peroxides and their possible breakdown products was desirable, and the TBA reaction was used as an estimate of hydroperoxide destruction (Dahle et al.1962).

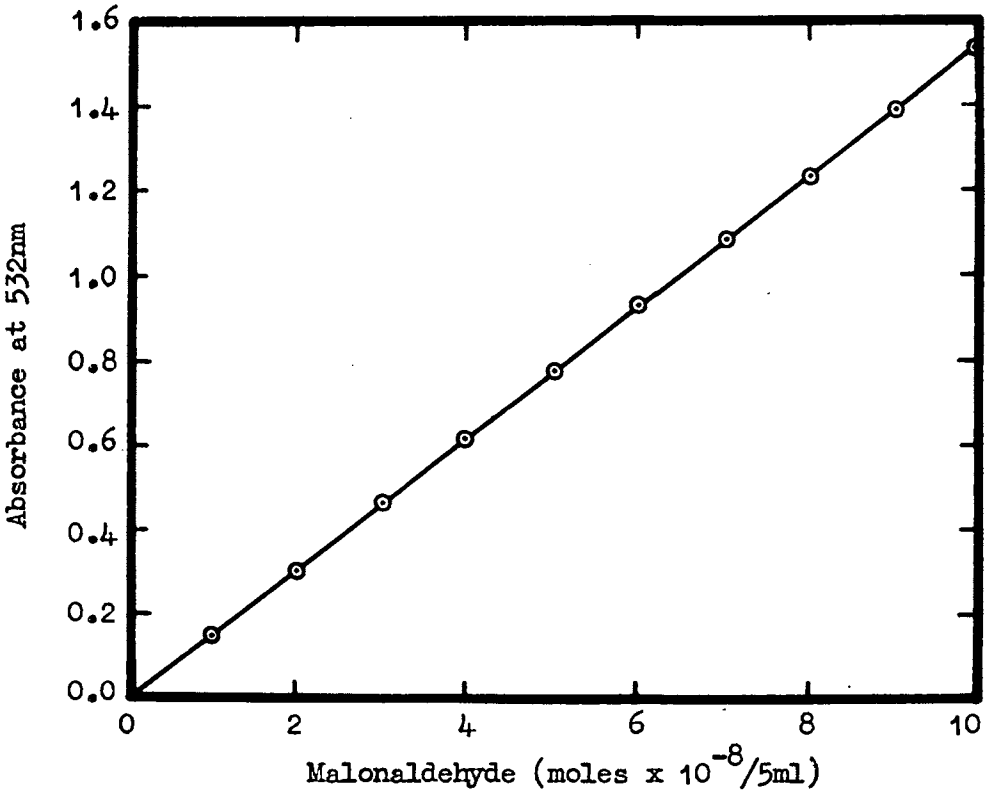
The TBA method finally adopted (Expt.22.13) was a modification of the distillation procedure of Tarladgis et al.(1960). The modifications were the use of 20g samples (instead of 10g) and the addition of sufficient hydrochloric acid to the sample before blending to produce a slurry of pH1.5. This step was particularly important in unblanched peas in which active lipoxygenase was present, and was used to prevent any lipid oxidation during the blending step. The modifications were similar to those described by Rhee and Watts (1966a). Rapid and constant distillation times at a constant pH were found necessary in order to obtain reproducible results, which were in agreement with the work of Tarladgis et al.(1960). Malonaldehyde concentrations were determined from absorbance readings of the TBA-malonaldehyde complex at 532nm (Graph 29), using a calibration curve (Graph 30) relating absorbance at 532nm to malonaldehyde concentration (Expt.22.11, Table 4.73). The malonaldehyde was formed by acid hydrolysis of 1,1,3,3-tetraethoxypropane (Sinnhuber and Yu 1958; Kwon and Watts 1963). The percent recovery of malonaldehyde from aqueous solutions by distillation at pH1.5 (Expt.22.12, Table 4.74) was found to average approximately 69.5%, slightly higher than the results of Tarladgis



GRAPH 29

Spectral curves of the condensation product of malonaldehyde with 2-thiobarbituric acid.

———	10×10^{-8}	moles	malonaldehyde/5ml.	
– – –	8×10^{-8}	"	"	"
–•• –	6×10^{-8}	"	"	"
•••••	4×10^{-8}	"	"	"
-•-•-•-	2×10^{-8}	"	"	"



GRAPH 30

Calibration curve for the estimation of malonaldehyde with 2-thiobarbituric acid.

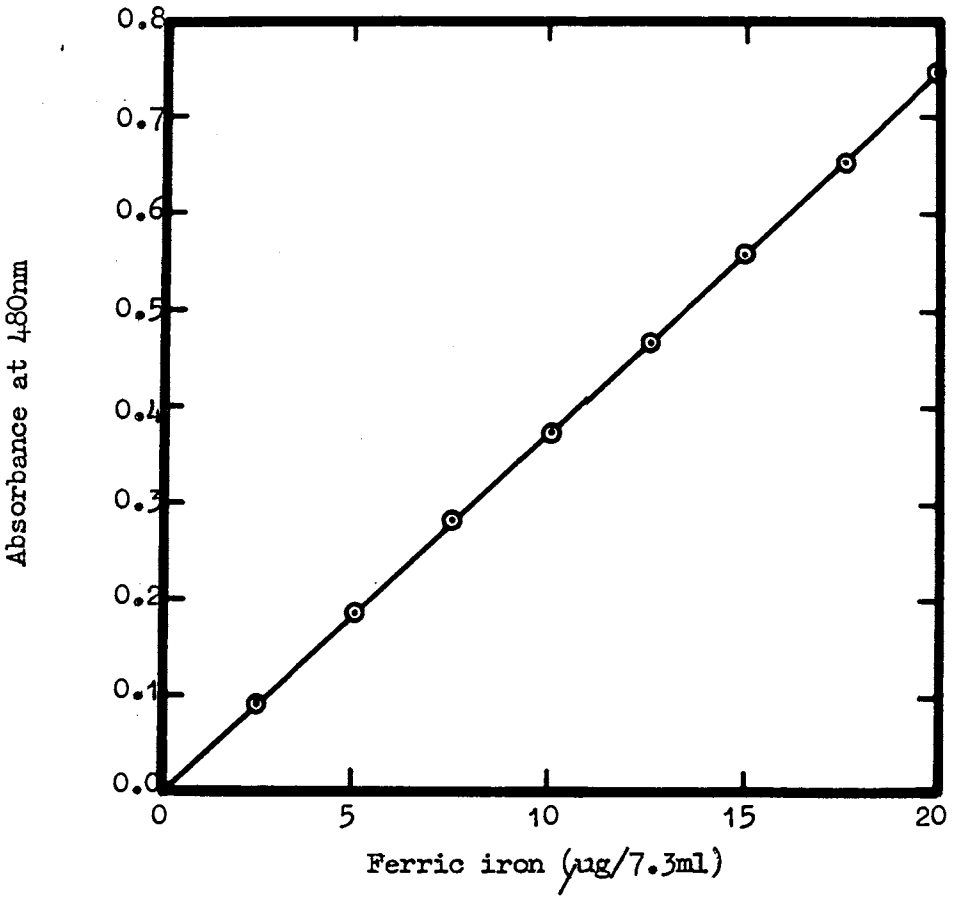
et al.(1960) and Kwon and Watts (1964). The distillation constant K, where $K = \frac{\text{TBA number}}{\text{absorbance at } 532\text{nm}}$, was found to be 3.338 for a 20g sample, somewhat lower than the value (3.9) reported by Tarladgis et al.(1960) and used by Rhee and Watts (1966a).

The second method used in this study for the estimation of fat oxidation was the ferric thiocyanate modification of Walker (1964a). Although the thiocyanate method has been shown to give abnormally high values in the presence of oxygen compared to iodometric peroxide values and oxygen absorption (Lea 1952), it nevertheless has been shown to be sensitive and reliable and to give meaningful results for comparative studies (Hills and Thiel 1946; Holloway 1966). Peroxide values (μ equivalents peroxide/100g material) were estimated on aliquots of chloroform lipid extract of peas and beans (Expt.22.24), after extraction of lipid material by a modification (Walker 1964a) of the method of Bligh and Dyer (1959) (Expt.22.21). Absorbance at 480nm of the ferric thiocyanate complex was recorded and ferric iron concentrations, obtained from a calibration curve (Expt.22.23, Table 4.76, Graph 31), were used to estimate peroxide values by the appropriate formulae (Expt.22.24). Great care was required in the use of blank solutions to compensate for absorption at 480nm not due to the ferric thiocyanate complex, e.g. pheophytins in the acid chloroform extract.

3.542 Lipid Oxidation During Frozen Storage

3.5421 Frozen Peas

The results of lipid oxidation determinations by the TBA and ferric thiocyanate methods on stored frozen vegetables are presented in Tables 4.75 and 4.77 respectively. Considering first the TBA values of peas, it

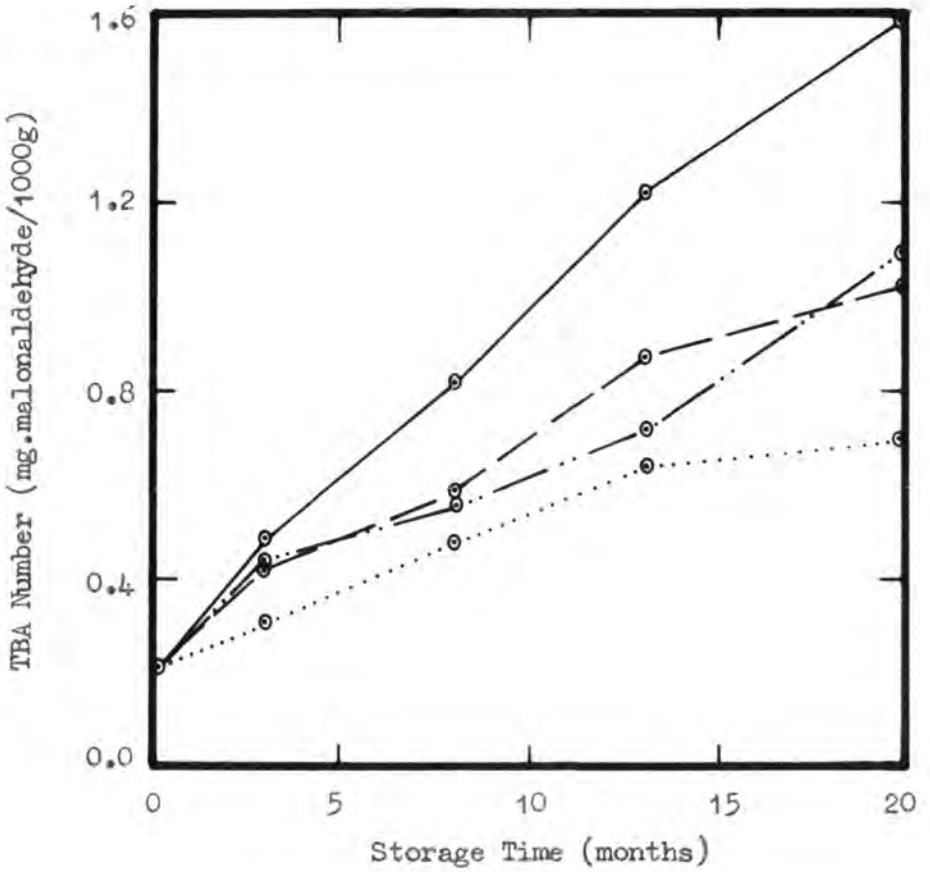


GRAPH 31

Calibration curve for the estimation of ferric iron with thiocyanate.

is seen that blanched peas showed a gradual increase in lipid oxidation with storage time at both temperatures, with values at 15°F slightly greater than at -10°F. Unblanched peas, however, showed a much more rapid increase in TBA number, particularly in the samples stored under oxygen (Graph 32). High values were found even at -10°F, showing that peroxide formation and subsequent peroxide breakdown was not prevented by the low temperature. Samples stored under nitrogen at both temperatures showed a slight levelling-off of the rate of formation of TBA-reactive substances. This could have been due to a lowering of the oxygen tension in the peas or to a decreased rate of hydroperoxide destruction.

The results for blanched and unblanched peas were in agreement with the observations of Rhee and Watts (1966b) in that relatively low TBA values were obtained compared with rancid animal products where TBA numbers may reach 300 or more. The samples of unblanched peas stored at 15°F had a pronounced off-colour, particularly those stored under oxygen. Unblanched peas stored at -10°F also possessed a marked off-odour compared to blanched samples stored at either temperature. The "storage" odours, however, were distinctly different from the odour of fresh unblanched peas blended vigorously with water in the presence of oxygen. The latter samples gave odours more typical of rancid, oxidised fats, while the stored pea odour was typical of a fruity, fermented, or spoiled product. These observations would tend to indicate that oxidation of fatty materials was not the principal cause of odour deterioration in frozen unblanched peas, in agreement with the conclusions of Rhee and Watts (1966b).

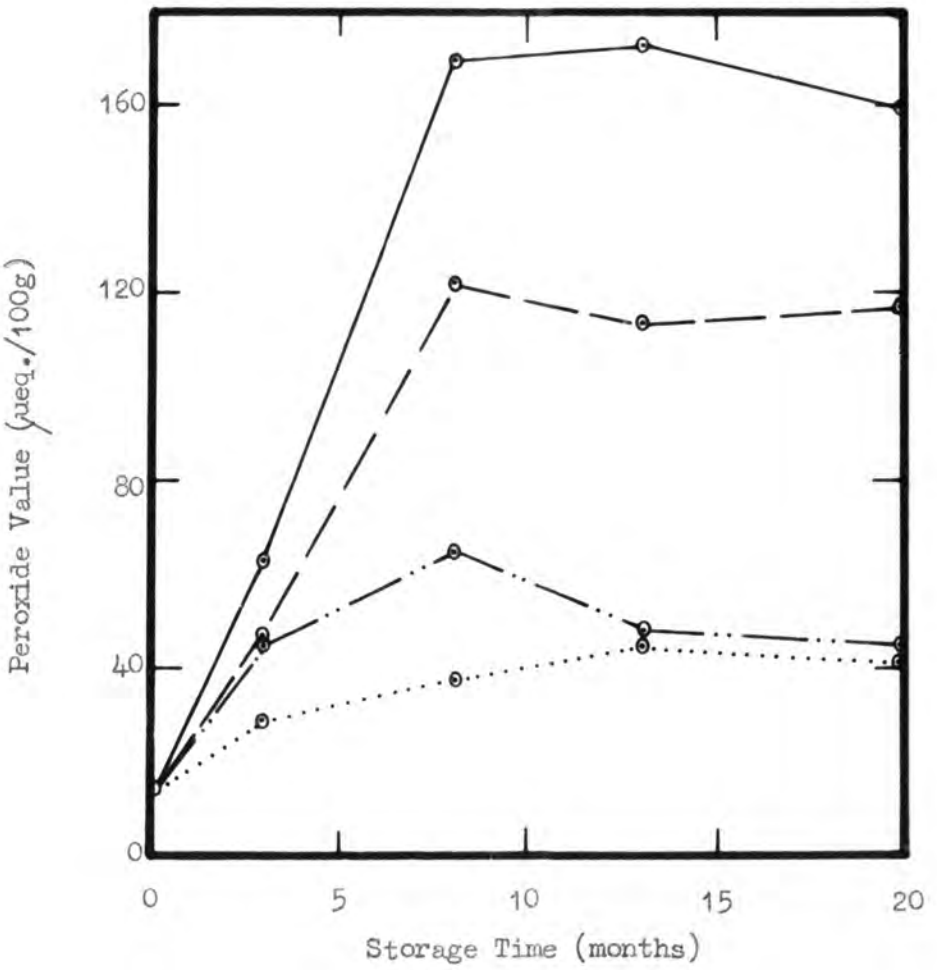


GRAPH 32

Changes in TBA number of frozen unblanched peas during storage.

——— Stored under oxygen at 15°F.
– – – " " nitrogen at 15°F.
–•–• " " oxygen at -10°F.
••••• " " nitrogen at -10°F.

Peroxide values of unblanched peas (Graph 33) showed similar trends to the TBA value up to 8 months storage time, but then decreased on further storage whereas the TBA values increased. The samples stored under oxygen at -10°F, which showed the largest decrease in peroxide value, also showed an increased rate of production of TBA-reactive substances during the later storage period, in agreement with the postulate that malonaldehyde or other TBA-reactive substances are secondary oxidation products formed by the breakdown of hydroperoxides (Dahle et al.1962). Although the initial rapid production of hydroperoxides and their subsequent slow disappearance in samples stored at 15°F can be correlated to some extent with the activity of lipoxygenase (Expt.34, Table 4.91), the peroxide contents of samples stored at -10°F are more difficult to explain since these samples, after storage for 8 months, showed little loss in lipoxygenase activity, yet the hydroperoxide content from that point either decreased or increased only slowly. The continued increase in TBA values without a parallel increase in peroxide value would indicate that either further peroxide formation was inhibited, possibly by the high peroxide concentrations accumulated, or a factor was present in the raw peas which destroyed peroxides faster than they were produced. Although such a factor was subsequently shown to be present in frozen unblanched peas (Section 3.63), it was destroyed at a faster rate than lipoxygenase during frozen storage. While it is possible that as the substrate (peroxide) concentration increased, the rate of peroxide destruction also increased, this would appear doubtful as the sample stored under oxygen at the lower temperature (-10°F) showed a greater peroxide loss than either of the samples stored at the higher temperature.



GRAPH 33

Changes in peroxide value of frozen unblanched peas during storage.

——— Stored under oxygen at 15°F.
– – – " " nitrogen at 15°F.
–·· – " " oxygen at -10°F.
····· " " nitrogen at -10°F.

3.5422 Frozen Beans

Tendergreen beans showed TBA results similar to that found for blanched peas with relatively slow increases at both temperatures and, after 20 months at 15°F, the TBA values had approximately doubled. Pearlgreen beans, however, showed substantially increased TBA values, particularly at 15°F, and over the 20 months storage period the values increased more than five fold. Even at -10°F, the TBA values of Pearlgreen beans doubled over the 20 month period. The TBA values of Pearlgreen-Brisbane beans were higher than unblanched peas stored at 15°F under nitrogen, or unblanched peas stored at -10°F under oxygen. No bean sample, however, showed distinctive off-odoursand were more acceptable than any of the unblanched peas.

Peroxide values of beans (Table 4.77) showed somewhat different results to TBA values. After 20 months at 15°F all bean samples gave relatively low peroxide values (μ equiv./100g) compared to unblanched peas, with the samples grown at Brisbane showing higher values than the Bathurst samples. When the results were converted to μ equiv./g lipid however, beans showed relatively high values, since the beans contained only about one fourth as much lipid as the peas. Highest values were obtained with the Brisbane samples of both varieties, whereas TBA numbers were higher for both Pearlgreen samples. The higher peroxide values of the Brisbane beans were in agreement with their slightly higher percent chlorophyll conversion and longer blanching time, and somewhat parallels the results of Walker (1964a,b). However, Walker used Tendergreen variety beans and reported that up to 12 months storage at 0°F, only small in-

creases occurred in fat peroxide (μ equiv./100g beans), but beyond 12 months, the peroxide increased from 31.2 to 172.9 μ equiv./100g up to 22 months storage. In the present study, peroxide values increased by a factor of less than four at 15°F, and the increases were relatively constant throughout the whole storage period.

The relatively high TBA numbers of Pearlgreen beans, compared to the Tendergreen variety, would tend to indicate that peroxides formed in the former samples were less stable than peroxides in the latter, yet a comparable difference in peroxide values was not apparent. There was, however, a slight drop in total pigment content in the samples showing high TBA values (Table 3.18). Possibly the Pearlgreen beans contained a higher content of linolenic acid compared to linoleic acid, since Dahle et al. (1962) has shown that peroxidation of the di-unsaturated acid does not yield TBA-reactive substances. However, the reported high concentration of both linoleic and linolenic acids in the lipids of beans (Wagenknecht 1963; Forman and Pokorny 1966) would make this assumption appear unreasonable. Although analyses for fatty acids of the two varieties could not be carried out in this work, the substantial difference in concentration of the two polyunsaturated acids required by this hypothesis would be most unlikely.

Although the process of lipid oxidation in unblanched peas can be postulated to be due to the presence of active lipoxygenase (Section 3.63), this explanation is untenable for the bean samples since they were blanched before freezing, and subsequently showed no enzyme activity during storage. Under these circumstances, free radical initiation and

chain propagation of lipid oxidation must have been induced by some other process, such as a more than adequate blanching treatment (Walker 1964b). No noticeable lag phase was found in this work before peroxide values and TBA values increased, in contrast to the results of Walker (1964a).

The substantial lipid oxidation found in blanched beans is in direct contrast to the small changes which occurred in blanched peas, even though the peas contained a considerably higher lipid content. This would indicate that bean lipids are inherently less stable than pea lipids. Whether this is due to the much higher proportion of linolenic acid compared to linoleic acid in beans compared to peas (Lee and Mattick 1961), or to the lower pH or some other factor, is uncertain but it has a considerable effect on the storage stability of the two frozen materials.

3.55 pH

All samples of peas were found to have higher pH values than any of the bean samples analysed (Expt.23, Table 4.78). Of the pea samples, those that were blanched showed a higher initial pH than unblanched samples, and during storage showed a significant increase in pH. The increase in pH as a result of blanching has also been found by other workers, and is probably caused by leaching of water soluble organic acids and the expulsion of intracellular gases such as carbon dioxide. The unblanched peas showed a gradual decrease in pH with increase in storage time, the decrease being more marked at 15°F than at -10°F. These results were in agreement with the higher chlorophyll conversion in unblanched samples, and would be caused in part by the enzymic

liberation of free fatty acids from fat glycerides during storage (Lee and Mattick 1961). There was no significant difference between samples stored under nitrogen and oxygen.

Even after 20 months storage at 15°F, unblanched peas showed a higher pH than the initial pH of all bean samples in which no enzymic activity was present. Tendergreen variety bean samples grown at both Bathurst and Brisbane were higher in pH than the Pearlgreen samples, but during storage at 15°F the Brisbane samples (Tendergreen and Pearlgreen) showed the greater decrease in pH. The low pH of beans was undoubtedly one of the factors contributing to their rapid pigment degradation during frozen storage.

3.6 CHLOROPHYLL BLEACHING AND LIPID OXIDATION IN MODEL SYSTEMS

Several reports in the literature have implicated a fat oxidation system in the bleaching of carotenoids (Blain et al.1953; Tookey et al. 1958) and the bleaching of chlorophyll pigments in systems containing unsaturated fatty acids and lipoxygenase (Wagenknecht et al.1952; Lee 1954; Mapson and Moustafa 1955; Wagenknecht and Lee 1956,1958; Walker 1964a,b; Holden 1965a,c,1967a,b; Bacon and Holden 1966). Walker (1964a) examined pigments and fat oxidation in stored frozen French beans and reported that chlorophylls were converted to pheophytins, and both chlorophylls and pheophytins were destroyed, during anaerobic and aerobic phases respectively of lipid oxidation occurring during frozen storage for 22 months at 0°F. In model system studies using fresh bean homogenate as a source of enzyme, Walker showed that crude chlorophyll preparations reacted differently to purified pigments, and that purified soybean

lipoxygenase had a similar effect to the crude bean enzyme with respect to degradation and destruction of pigment.

Some of Walker's results were not substantiated by the work of Holden (1965a) who examined chlorophyll bleaching in extracts from legumes and legume seeds. In these studies, pure lipoxygenase and unsaturated fatty acids did not bleach chlorophyll without the addition of a "bleaching factor" present in the crude extracts. This factor had properties similar to a lipohydroperoxidase previously reported in soya extracts (Blain and Styles 1959; Blain and Barr 1961; Gini and Koch 1961) and the bleaching was considered to be the result of pigment destruction by hydroperoxide breakdown products formed enzymically by the "bleaching factor". The factor was different from lipoxygenase, present in high concentrations in soya extracts, which produced hydroperoxides from specific polyunsaturated fatty acids. The formation of hydroperoxides in the absence of the "bleaching factor" did not bleach chlorophyll, showing the mechanism to be different from the coupled bleaching of carotenoids (Blain et al.1953).

Since the initial part of the work on frozen materials was a storage trial in which pigments and lipid oxidation were measured, it was considered desirable to analyse enzyme systems responsible for some of the changes reported in the literature, particularly in unblanched samples. Consequently, storage analyses on peas and beans included determinations of bleaching activity and lipoxygenase activity. Model system studies using soya and fresh pea enzyme extracts were carried out to determine the importance, if any, of chlorophyll bleaching in the loss of colour during storage of frozen vegetables. The model system studies with soya

and pea enzyme systems are discussed first and these results are used to correlate enzymic reactions with pigment, colour and lipid oxidation changes during storage.

3.61 Experiments with Soya Extracts

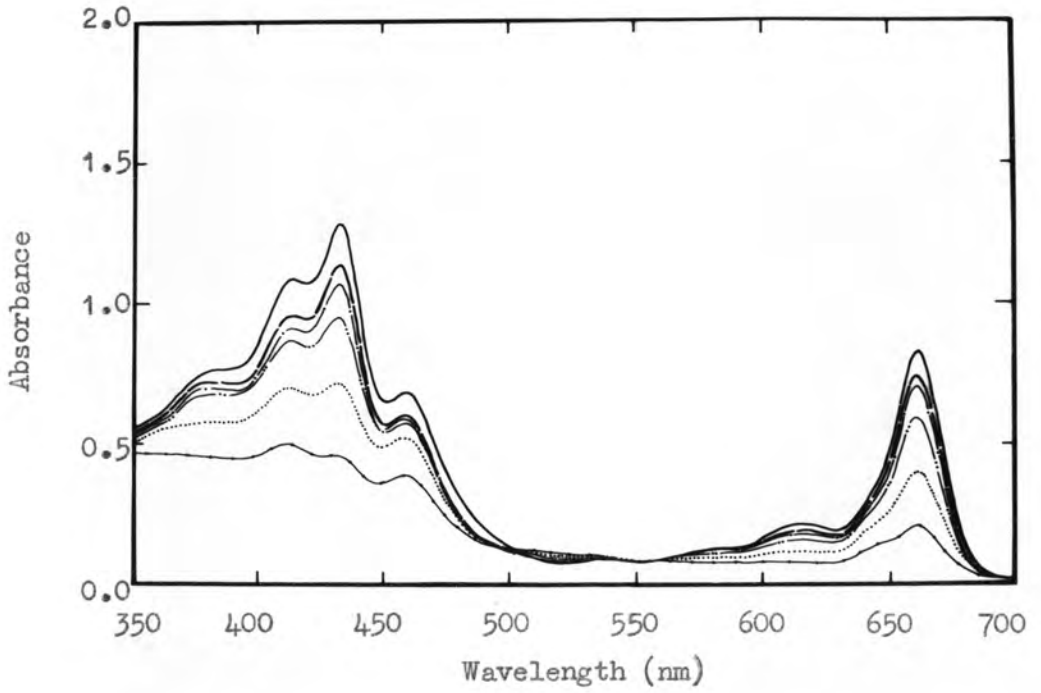
Preliminary experiments were carried out on extracts of soyabean meal to compare part of the present work with the results of Holden, Walker and others. The activity of bleaching enzyme systems was determined (Expt.24) by methods described by Holden (1965a) in which chlorophyll, pure unsaturated fatty acids (e.g. linoleic acid) and enzyme solutions were incubated under standard conditions for various reaction times, and the loss recorded of chlorophyll compared to suitable control solutions.

The effect of pH on the bleaching activity of soyabean meal extract was determined by incubating enzyme solutions with crude chlorophyll solution, linoleic acid and buffer solutions (0.1M or 0.2M) of pH4.5 to pH9.0 (Expt.25). The results (Table 4.79) showed that under the incubation conditions employed, more chlorophyll bleaching occurred at pH6.0 to 7.0 than at more acid or alkaline values. In the absence of added linoleic acid, more chlorophyll bleaching occurred at pH6.0 to 6.5 and this effect was more noticeable after the longer incubation time. With the addition of 5mg pure linoleic acid, percent chlorophyll bleaching was higher at pH6.5 to 7.0. The shift to a slightly higher pH optimum on the addition of acid was in agreement with the results of Holden (1965a), although pH optima somewhat lower were reported in that work using extracts from soya beans and veronal-acetate buffers. The pH values at which maximum chlorophyll bleaching were recorded in the present

work were not necessarily the true pH optima for the bleaching enzyme system, since at low pH values, availability of substrate would become a limiting factor. Only the solution containing pH9.0 borate buffer was optically clear after incubation, the remaining solutions being turbid due to pigment and acid in colloidal suspension. A pH of approximately 6.5 therefore represented the acidity at which enzyme activation and substrate solubility were at a combined maximum under the conditions used. At higher pH values, it would be expected that hydroperoxide production would increase because of the activity of lipoxygenase at alkaline pH values in the absence of detergent (Surrey 1964; Ames and King 1966) and the increased solubility of linoleic acid. The observation that chlorophyll bleaching did not increase with higher pH values is in agreement with the hypothesis that hydroperoxide formation alone is insufficient for chlorophyll destruction.

The visible spectra of bleached crude chlorophyll solutions at pH 6.5 are shown in Graph 34. It will be observed that on the addition of linoleic acid there was some conversion to pheophytin. This was particularly noticeable in the control solutions containing chlorophyll, linoleic acid and heat-inactivated enzyme solution. The rapid decrease in absorbance at 432nm and 662nm compared to the smaller absorbance changes at 456nm and 645nm is in agreement withprevious observations that chlorophyll a is degraded more rapidly than chlorophyll b.

The addition of purified soybean lipoxygenase to a solution containing chlorophyll and soya extract at pH6.5 (Expt.26, Table 4.80, Graph 35), increased only slightly the bleaching produced by crude soya



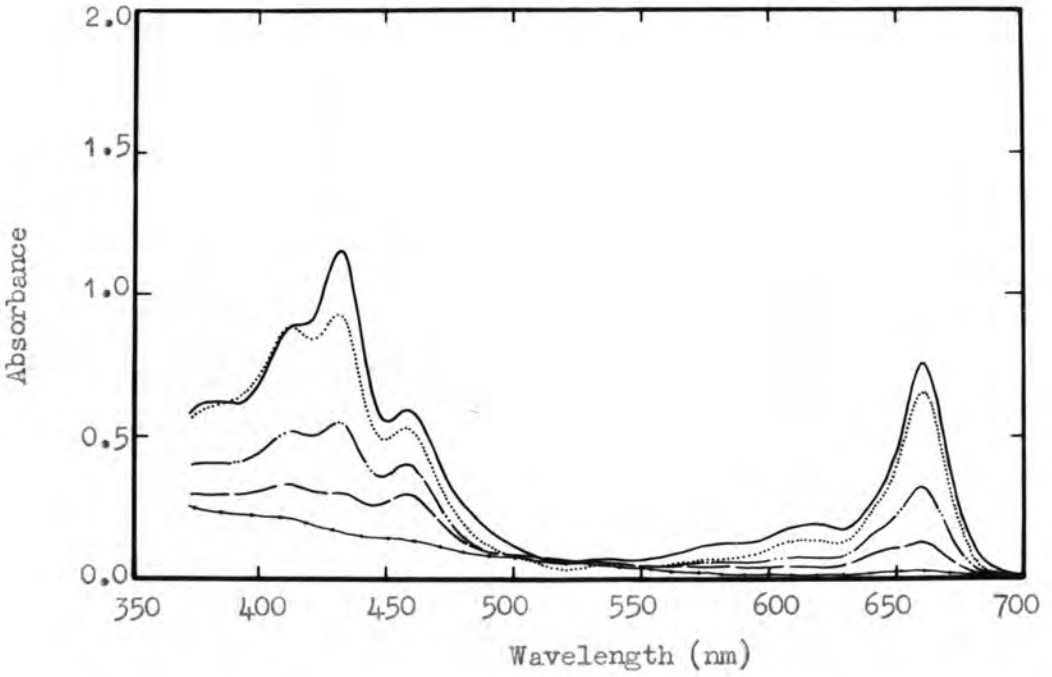
GRAPH 34

Bleaching of crude chlorophyll solution at pH 6.5 with soyabean extract and linoleic acid.

——— No extract, no linoleic acid, 1 min. incubation.
– – – Extract , " " " , 2 " " .
—•—•— " , " " " , 4 " " .
— ••— " , linoleic acid , 1 " " .
••••• " , " " , 2 " " .
•••••• " , " " , 4 " " .

extract alone showing the limiting factor in the crude enzyme extract to be unsaturated fatty acid, and not lipoxygenase enzyme. The addition of pure lipoxygenase as well as linoleic acid to crude soya extract further increased the bleaching of chlorophyll. Incubation of pure lipoxygenase and linoleic acid without the addition of crude extract showed negligible bleaching, indicating a factor in the extract to be primarily responsible for breakdown of pigment. These results were in agreement with the work of Holden (1965a) but did not substantiate the claim by Walker (1964a) that pure soybean lipoxygenase produced the same bleaching effect as a crude (bean) enzyme extract.

If it is postulated that hydroperoxides produced by the action of lipoxygenase on specific polyunsaturated fatty acids are destroyed by the "bleaching factor", then the addition of fatty acids which are not substrates for lipoxygenase would be expected to result in no bleaching over and above that caused by substrate fatty acids present in the crude enzyme solution. Lipoxygenase has been shown to catalyse the oxidation by molecular oxygen of polyunsaturated fatty acids containing a cis-cis 1,4-pentadiene system such as linoleic, linolenic and arachidonic acids, esters and glycerides, but not stearic, oleic or elaidic acids. However, the incubation of stearic, oleic and elaidic acids with chlorophyll and crude soya extract (Expt.27, Table 4.81), increased chlorophyll bleaching over a control containing no added fatty acid. This was in agreement with the work of Holden (1965a), but the bleaching with non-substrate fatty acids in the present work was much lower than that found by Holden, who reported that with oleic and elaidic acids, and to a lesser extent



GRAPH 35

Effect of purified lipoxygenase on chlorophyll bleaching with soyabean extract and linoleic acid.

——— Linoleic acid, 4 min. incubation.
– – – Soya extract, 1mg lipoxygenase, linoleic acid, 2 min. incubation.
– ·– " " , linoleic acid, 2 min. incubation.
····· Linoleic acid, 2mg lipoxygenase, 4 min. incubation.
~~~~~ Soya extract, linoleic acid, 1mg lipoxygenase, 4 min. incubation.
~~~~~

stearic acid, chlorophyll was bleached in acetate buffer, pH5.9, to the same extent as with arachidonic acid and only slightly less than with linoleic and linolenic acids. The higher bleaching with the all-cis unsaturated acids shown in Table 4.81, compared to the published results, may have been due to the use of acids of higher purity, or to different incubation conditions such as pH and enzyme activity. Since some bleaching was produced without the addition of any fatty acid, this showed that unsaturated acids were present in the soya extract. It is possible that peroxidation of these acids and subsequent enzymic destruction of the hydroperoxides formed may have induced free radical autoxidation in the non-substrate fatty acids, thus resulting in an increase in bleaching. The addition of pure lipoxygenase to oleic, elaidic or stearic acids under similar conditions without the presence of soya extract showed no bleaching at all. The similar extent of bleaching produced by the addition of linoleic, linolenic or arachidonic acids was in agreement with previous observations (Siddiqi and Tappel 1957) that these substrates are oxidised by lipoxygenase at equal rates in great contrast to their comparative behaviour in autoxidation, providing it is assumed that the different hydroperoxides produced were subsequently also broken down at equal rates.

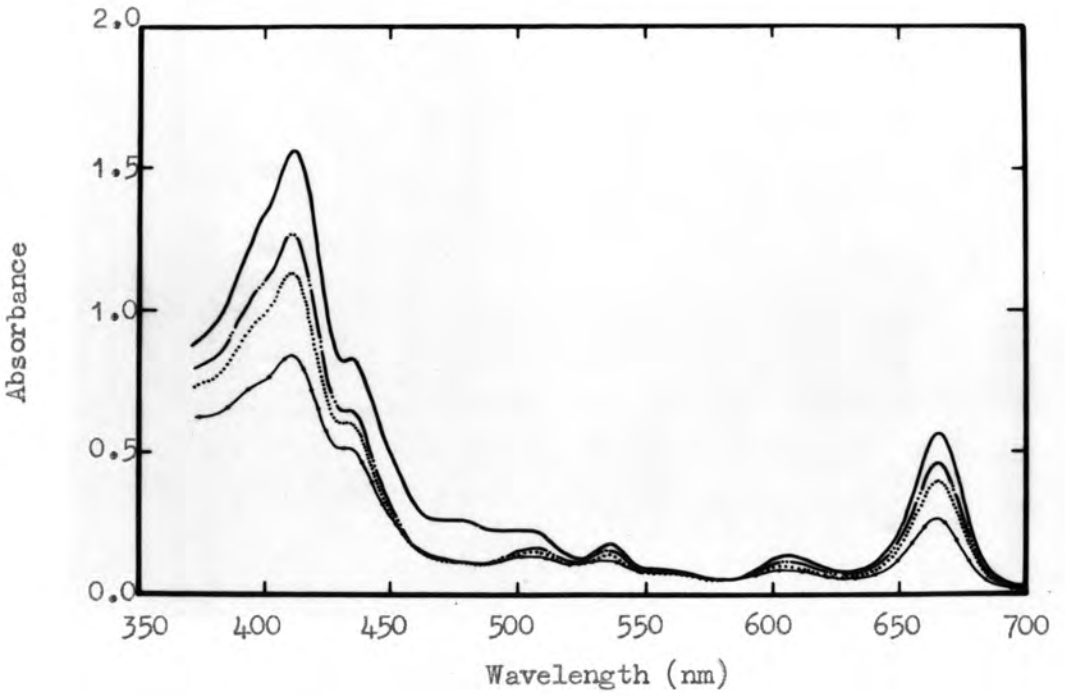
Purified chlorophylls a and b were bleached by soya extract to a much lesser degree than either crude chlorophyll or pheophytin solutions (Expt.28, Table 4.82). The addition of linoleic acid also produced smaller increases in bleaching when added to pure pigment solutions. This effect was possibly due to the inability of purified pigments to remain in colloidal suspension since some phase separation was evident

unless the solutions were vigorously shaken. Crude pigment solutions showed no phase separation and remained in colloidal suspension for long periods of incubation. Mg-free pigments were less susceptible to bleaching than the chlorophylls, particularly after the addition of linoleic acid. Pheophytin a was also degraded faster than pheophytin b (Graph 36), as was chlorophyll a compared to chlorophyll b (Graph 34, Table 4.82). The lower percent bleaching observed with pure pigments does raise the possibility, however, that factors in the crude pigment extract, such as carotenoid pigments, accelerate the bleaching reaction. For example, it is known that β-carotene and other carotenoids are bleached in lipoxygenase-linoleate systems (Blain et al.1953) and by the decomposition of organic hydroperoxides (Elahi 1967) due to hydrogen abstraction and free radical oxidation (Siddiqi and Tappel 1957).

3.62 Experiments with Pea Extracts

The preceding experiments showed that under the conditions used in the present work, chlorophyll bleaching could be produced that was essentially similar to that described by Holden (1965a). In an attempt to correlate previous storage studies with possible mechanisms of pigment destruction, experiments were carried out on extracts from fresh peas, and where possible results were compared to those obtained from analyses of stored frozen vegetables.

The presence of chlorophyll bleaching activity was examined (Expt. 29) in extracts of fresh peas, in an acetone powder prepared from fresh peas, and in pea seeds. The results (Table 4.83) showed that comparable bleaching activity was present in extracts of fresh peas and pea seeds



GRAPH 36

Bleaching of crude pheophytin solution with soyabean extract and linoleic acid.

——— Linoleic acid, 2 min. incubation.

—•• — Soya extract, no linoleic acid, 4 min. incubation.

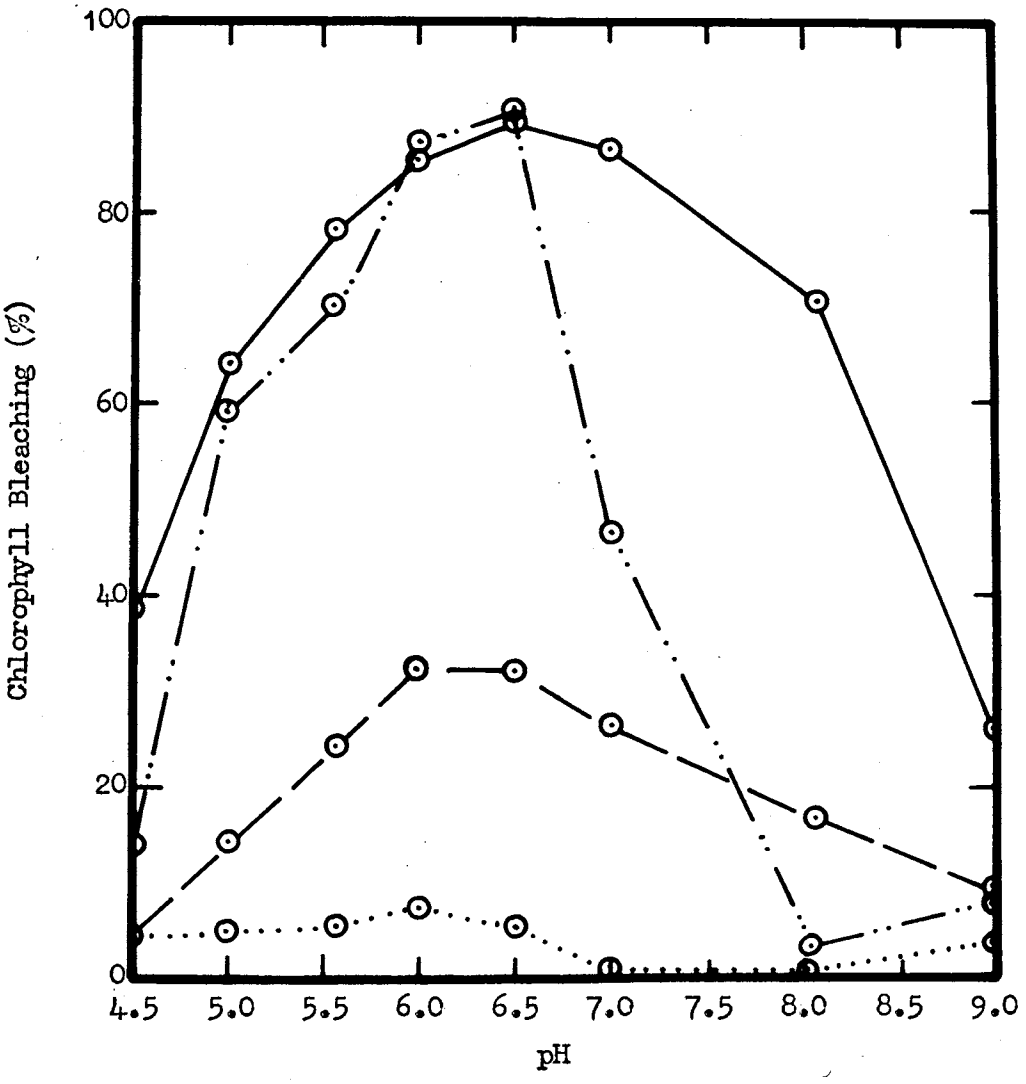
••••• " " , linoleic acid , 2 " " .

•••••• " " , " " , 4 " " .

at the concentration levels used, but no activity at all was present in the acetone powder. Enzyme inhibition or inactivation in the presence of high concentrations of acetone was also found by Holden (1965a). Subsequent experiments not reported in this work showed that considerable lipoxygenase activity was retained in acetone powders of fresh peas in agreement with published results (Mapson and Moustafa 1955). The lower stability of the bleaching factor compared to lipoxygenase was also found in stored frozen unblanched peas (Expt.34).

The effects of pH on the bleaching of chlorophyll by pea extract (Expt.30) are shown in Table 4.84. Optimum pH for bleaching with and without added linoleic acid were approximately pH6.5 and pH6.0 respectively, slightly lower than that observed with crude soya extract. However, chlorophyll bleaching by pea extract in the presence of linoleic acid occurred over a much narrower pH range than with soya extract (Graph 37). Activity dropped sharply above pH6.5-7.0 and below pH5.0. It was also observed that the addition of linoleic acid showed a substantial increase in bleaching with pea extract, much greater than the corresponding increase with crude soya extract. This would indicate that soya extracts contained more polyunsaturated fatty acids than did pea extract, rather than higher concentrations of lipoxygenase or bleaching factor.

The effects of increased enzyme concentration and incubation time on chlorophyll bleaching by pea extract (Expt.31.1) are presented in Table 4.85 and shown on Graph 38. With a small volume of enzyme added (0.05ml), bleaching was proportional to incubation time. At higher enzyme levels, the rate of bleaching decreased with increased incubation



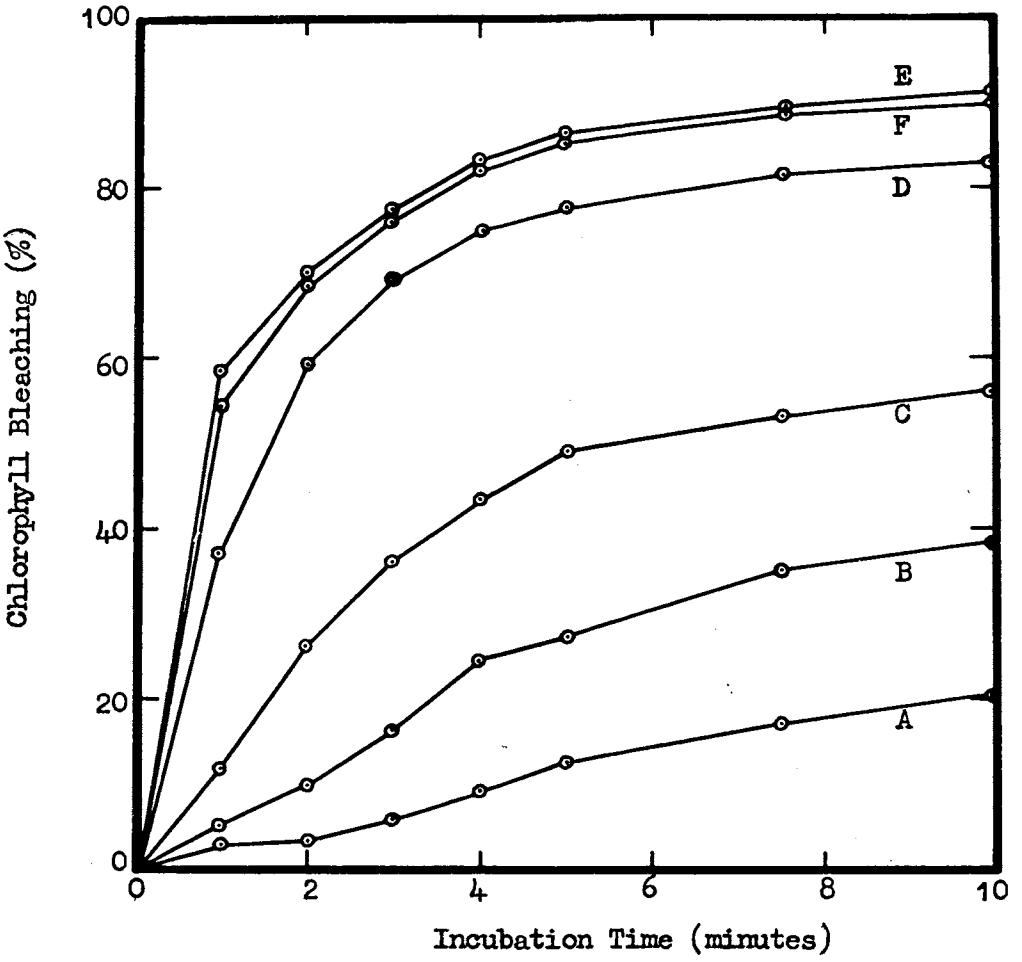
GRAPH 37

Effect of pH and linoleic acid on chlorophyll bleaching with soya and pea extracts.

——— Soya extract, linoleic acid, 4 min. incubation.
– – – " " , no " " , 4 " " .
–·· – Pea extract , linoleic acid, 4 " " .
····· " " , no " " , 4 " " .

time similar to other enzyme systems. At a higher enzyme level (1.0ml), more than half the total bleaching occurred in the first minute of incubation, but no further increase in bleaching was observed with the highest enzyme concentration (2.0ml). This would indicate that the enzymic reactions involved (lipoxygenase activity or bleaching activity) were working to maximum capacity, or possibly that, as a result of initial reactions, inhibitory substances were produced which inactivated the bleaching enzyme(s) present. The results may also be interpreted as evidence that the bleaching enzymes were present in low concentrations in the pea extract, and only at high enzyme levels was sufficient activity present to enable rapid hydroperoxide breakdown to occur over a short incubation period.

The latter alternative appears to be substantiated by the results presented in Table 4.86 showing ultra-violet absorption of linoleic acid solutions incubated with pea extract (Expt.31.21). Absorbance at 234nm in 60% alcohol, due principally to conjugated hydroperoxides of the type $-CH_2-CH(OOH)-CH=CH-CH=CH-CH_2-$, increased substantially even at low (0.05ml) enzyme levels, showing the presence of considerable lipoxygenase activity. At all enzyme levels, absorbance at 234nm increased with increasing incubation time up to 10 minutes. A similar increase was observed at 1 minute and 2 minute incubation periods with increasing enzyme concentrations up to 1.0ml. However, after 5 minute and 10 minute incubation periods, absorbance at 234nm decreased slightly at high (1.0ml) enzyme levels, indicating the net loss of conjugated structures. Hydroperoxide breakdown was not confined to long incubation times and high enzyme



GRAPH 38

Effect of incubation time and enzyme level on chlorophyll bleaching with pea extract.

A	0.05ml	pea extract
B	0.1ml	" "
C	0.2ml	" "
D	0.5ml	" "
E	1.0ml	" "
F	2.0ml	" "

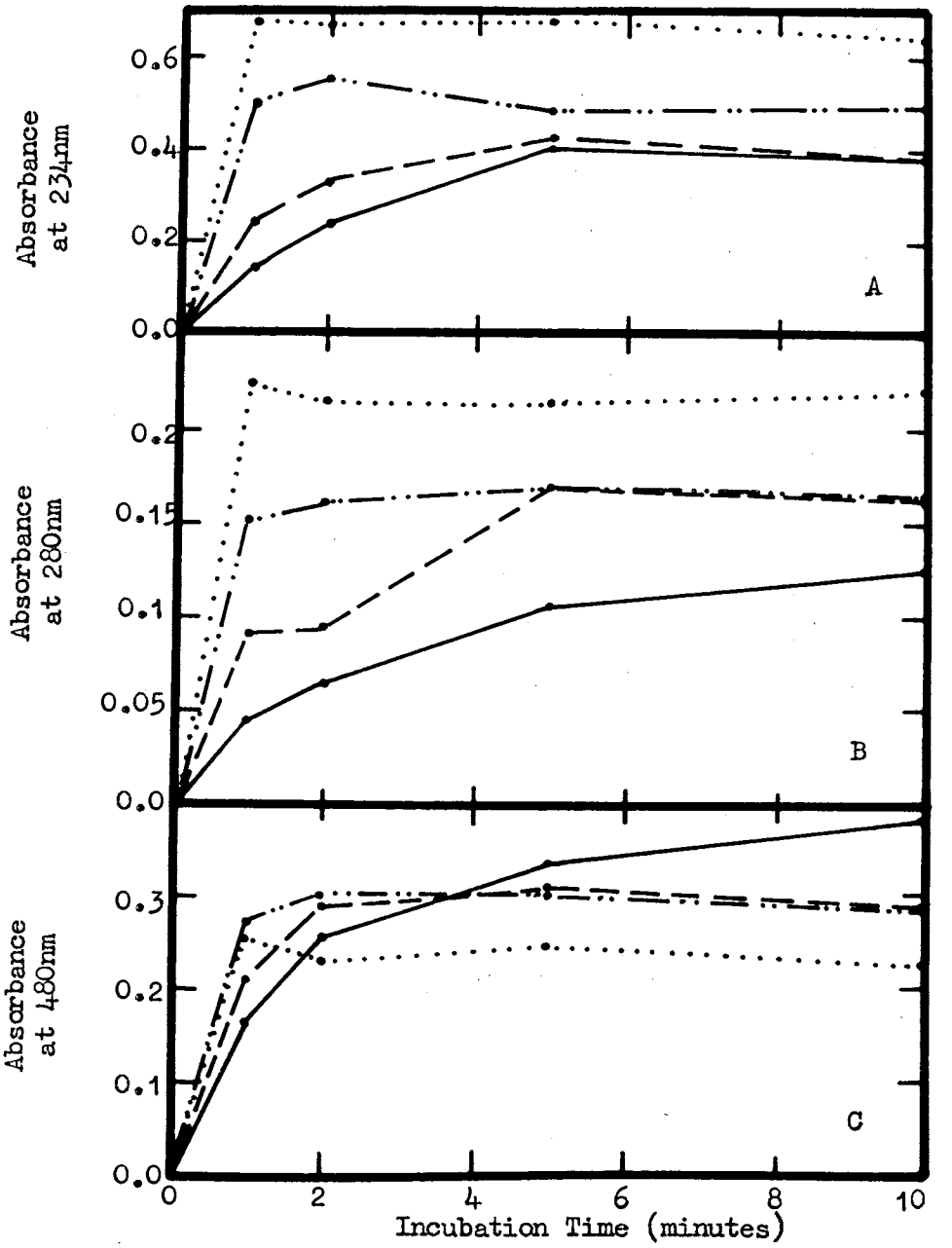
levels, but occurred in all incubations as shown by absorbance at 280nm (Table 4.86). The absorption at 280nm has been attributed to the presence of carbonyl compounds and/or conjugated trienes (Siddiqi and Tappel 1956), and an increase in absorbance near 280nm is indicative of hydroperoxide breakdown. Absorbance at 280nm increased with increases in incubation time and enzyme level, but was particularly significant at higher enzyme levels and incubation times. The increases in absorbance at 234nm did not parallel the increases at 280nm, which supports the hypothesis that peroxide formation (A_{234}) and peroxide destruction (A_{280}) are produced by different reactions.

The absorption at 234nm is primarily due to conjugated dienes, in particular, conjugated hydroperoxides. However, it is possible that hydroperoxide breakdown products also retained conjugated chromophores giving no net decrease in ultraviolet absorption at 234nm. The production of carbonyls, conjugated trienes or other polymeric substances would, nevertheless, increase the absorption near 280nm and give a clear indication that hydroperoxide breakdown had occurred.

In order to compare the above results with previous observations on hydroperoxide production and breakdown in soya extracts, similar experiments were carried out (Expt.31.22) using crude soyabean meal extract and linoleic acid. In addition to ultraviolet absorption at 234nm and 280nm, hydroperoxides were estimated by ferric thiocyanate absorption at 480nm (Table 4.87, Graph 39). Results showed correlations under some conditions, but opposite trends after long incubation periods. Absorbance at 234nm increased at each incubation period with increasing

GRAPH 39

Formation of hydroperoxides from linoleic acid incubated with soya extract.



A UV absorption at 234nm ——— 0.1ml soya extract
B UV " " 280nm - - - 0.2ml " "
-•-• 0.5ml " "
C FeSCN " " 480nm •••• 1.0ml " "

enzyme level, and showed no decreases as observed with pea extract. However, considering each enzyme level separately, absorbance at 234nm increased up to 5 minutes and then decreased for 0.1ml and 0.2ml enzyme levels, whereas for the 0.5ml enzyme level absorbance decreased after 2 minutes incubation, and at the highest enzyme level(1.0ml), maximum absorbance at 234nm was obtained after 1 minute incubation, and then the absorbance slowly decreased. Overall, it was apparent that as the level of enzyme was increased, the shorter was the elapsed time of incubation before the content of conjugated diene decreased, a result in agreement with the low initial concentration of bleaching factor or hydroperoxide breakdown factor.

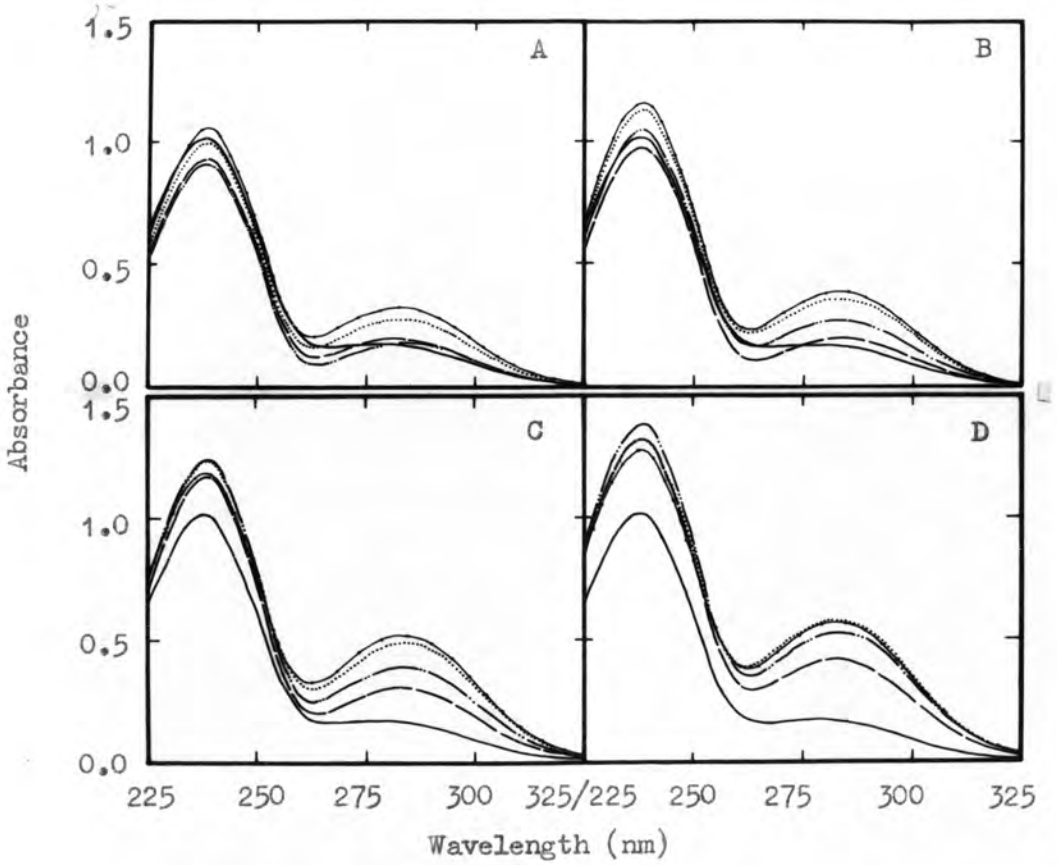
Considering the ferric thiocyanate absorption, similar absorbance changes were observed for each enzyme level with increasing incubation time as was observed for absorption at 234nm. The result of increasing the enzyme concentration separately for each incubation period was the reverse of that observed by absorption at 234nm, since at the longer incubation times (5 and 10 minutes), maximum peroxides were formed with 0.1ml enzyme, and higher enzyme levels resulted in further destruction of peroxide. These results indicated that although the hydroperoxide content decreased, some of the breakdown products must have retained the conjugated diene structure and thus contributed to the increasing absorbance at 234nm. Peroxide decomposition was continuous throughout the incubation period since absorption at 280nm increased at all enzyme levels.

The initial rapid formation of peroxides and their subsequent

destruction with high enzyme concentrations was in agreement with previous work on soya extracts (Blain and Styles 1959; Blain and Barr 1961; Gini and Koch 1961; Holden 1965a), and the results observed in pea extracts indicated the presence of a similar factor in peas. To gain further insight into the possible role of a lipohydroperoxidase-type factor in pea extracts, experiments were carried out with linoleic acid previously oxidised with pure lipoxygenase (Expt.32.1). Oxidised linoleic acid showed a prominent peak at 234nm, but only a minor plateau near 280nm, indicating the absence of high concentrations of hydroperoxide breakdown products (Graph 40). Oxidised linoleic acid did not bleach crude chlorophyll solution without the addition of active pea extract (Expt.32.2, Table 4.88) showing that hydroperoxides alone were insufficient for pigment bleaching. This finding substantiates the claim that peroxide destruction is responsible for chlorophyll bleaching. Normal linoleic acid also showed no effect without the addition of crude enzyme. In the presence of pea extract and oxidised linoleic acid, chlorophyll was bleached to the same extent as with normal linoleic acid during short incubation periods, but on further incubation, the bleaching rate decreased compared to that produced by unoxidised acid. The same result was produced when the experiment was repeated with different samples of oxidised linoleic acid, and different enzyme extracts (Table 4.90), and it would appear that in the presence of very high concentrations of hydroperoxides, or peroxide breakdown products, the bleaching enzyme(s) was partially inactivated. This is not unexpected as peroxidised lipids and their breakdown products have been shown to produce protein denatur-

ation and enzyme inactivation (Kokatnur et al.1966; O'Brien and Fraser 1966; Roubal and Tappel 1966).

Ultraviolet absorption of oxidised linoleic acid incubated with pea enzyme (Expt.32.3) showed results in fair agreement with previous observations (Table 4.89, Graph 40). Absorbance at 234nm showed unusual trends in that low enzyme levels (0.1 and 0.2ml) produced an initial decrease followed by an increase to above the blank determination with increased incubation time, while higher levels (0.5 and 1.0ml) showed an initial increase followed by a decrease after 2 minutes incubation. A decrease in absorbance at the 0.1ml enzyme level was also observed for each incubation period from 1 to 10 minutes. At short incubation periods (1 and 2 minutes), the absorbance decreased to a value lower than the blank solution containing acid but no enzyme, while at longer times (5 and 10 minutes), the lower value was not below that of the control. Although these results appear to contradict previous observations, it should be remembered that the observed absorbance at 234nm was due to (1) preformed peroxide, (2) additional peroxide produced by the action of lipoxygenase on unoxidised linoleic acid, and (3) hydroperoxide decomposition products retaining a conjugated diene structure. It is significant, however, that in all cases, ultraviolet absorption near 280nm increased with enzyme level and incubation time, and at no stage fell below that of the control solutions. It is of interest to note also that the lower the enzyme level, the longer the incubation time necessary for a noticeable increase in absorbance at 280nm. Similarly, the shorter the incubation time, the greater the enzyme



GRAPH 40

Effect of incubation time and level of pea extract on breakdown of linoleic acid hydroperoxides.

A	0.1ml pea extract	———	0 min. incubation
B	0.2ml " "	– – –	1 " "
C	0.5ml " "	– • –	2 " "
D	1.0ml " "	·····	5 " "
		▲▲▲▲▲	10 " "

concentration required for a significant increase in absorbance. These latter results show quite clearly that hydroperoxide breakdown occurred at all enzyme levels, but was considerable only at higher enzyme levels, in agreement with the previous observations that the bleaching factor is present in relatively low concentrations in crude extracts.

The hypothesis that chlorophyll bleaching is caused by hydroperoxide decomposition catalysed by the enzymic bleaching factor, should make it possible to bleach chlorophyll in a system containing preformed hydroperoxide and a peroxide-decomposition catalyst. An obvious choice of reagent to catalyse the breakdown of peroxides are the hematin compounds, which have been clearly shown to catalyse the oxidation of unsaturated fatty acids as well as the destruction of hydroperoxides (Tappel 1961, 1962b). Consequently, oxidised linoleic acid was incubated with pea enzyme and the hematin compound hemin (final concentration 2×10^{-6} Molar), and chlorophyll bleaching determined (Expt.33, Table 4.90). No bleaching was observed in flasks containing normal or oxidised linoleic acid and heat-inactivated pea extract, or normal linoleic acid and hemin solution. When hemin was added to oxidised linoleic acid and chlorophyll, the pigment was bleached to an extent comparable to that with normal acid and pea extract up to 1 minute incubation. In contrast to the enzyme-catalysed reaction, hemin-catalysed bleaching showed little further increase with incubation, and was probably due to the destruction of hemin by hydroperoxides or hydroperoxide breakdown products (Kokatnur et al.1966).

The addition of hemin and pea extract to both normal and oxidised linoleic acid caused a marked initial increase in bleaching over that

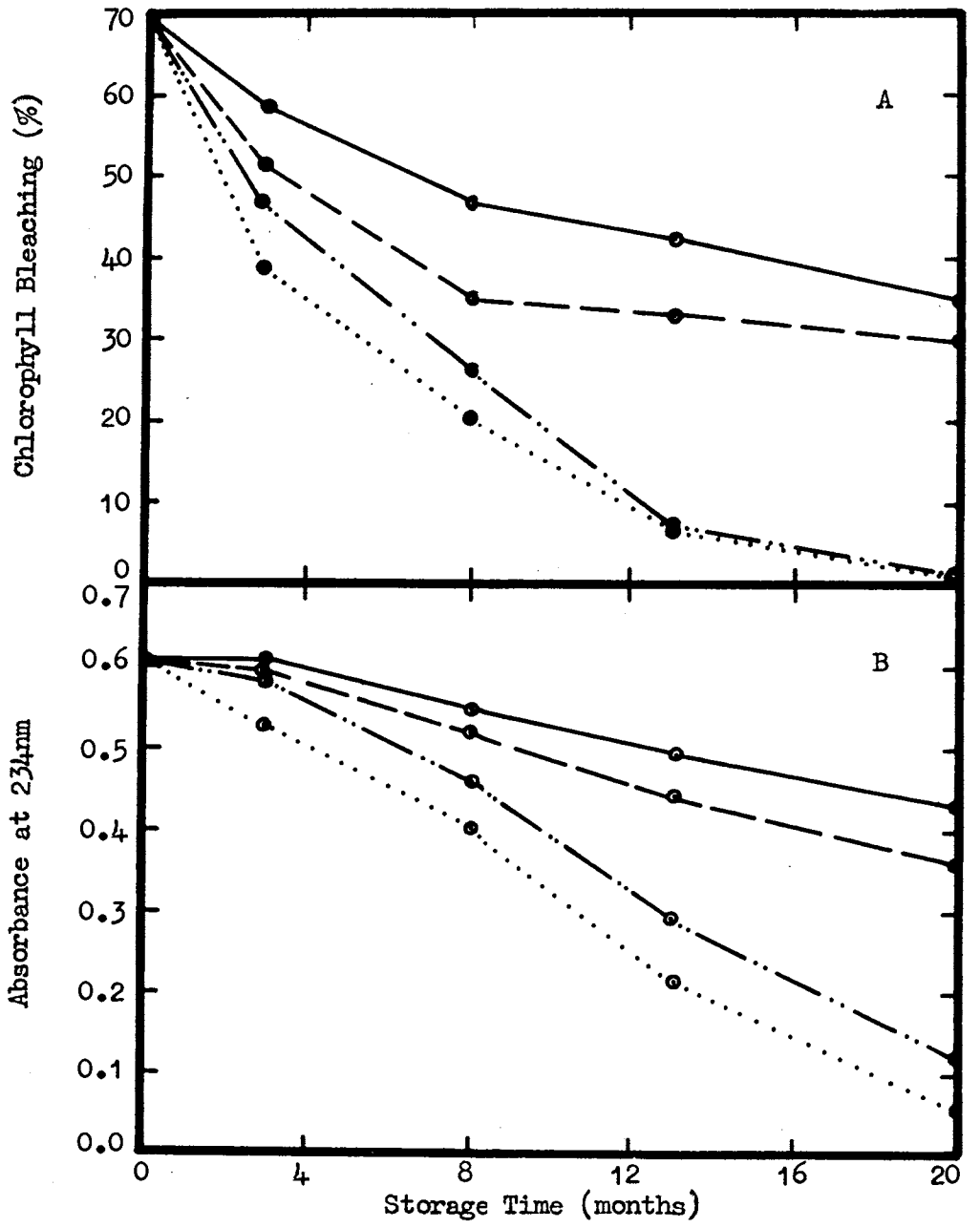
produced by pea extract and normal or oxidised acid, but on further incubation, this initial increase was not retained, since after 10 minutes incubation, solutions containing hemin showed only slightly higher bleaching than those without hemin. These results can be explained by the decomposition of preformed hydroperoxides during the early incubation stage, thereby increasing chlorophyll bleaching and the subsequent loss or decrease of catalytic activity of hemin and the bleaching factor(s) by hydroperoxides or their decomposition products. The results of these experiments are thus in keeping with the postulate that hydroperoxide decomposition is responsible for chlorophyll bleaching since (1) hydroperoxides alone did not bleach chlorophyll, (2) bleaching was produced when hydroperoxides were catalytically decomposed by hemin, (3) preformed hydroperoxides plus pea extract bleached chlorophyll, (4) hemin increased bleaching over that produced by pea extract and preformed hydroperoxide, (5) hemin increased bleaching over that produced by pea extract and normal linoleic acid, (6) absorption at 280nm increased when bleaching occurred.

3.63 Chlorophyll Bleaching and Lipoxygenase Activities in Stored Frozen Vegetables

It is apparent from the previous section that chlorophyll bleaching by extracts from fresh peas occurred by the reaction with pigment molecules of free radicals or other species produced by decomposition of hydroperoxides. Since bleaching resulted in the loss of total pigment, it was of interest to establish whether this type of pigment destruction was responsible for the loss of total pigment during the storage of frozen unblanched peas (Table 3.16). Results of chlorophyll bleaching

activity and lipoxygenase activity (Expt.34, Table 4.91) in extracts of unblanched peas stored for periods up to 20 months at 15°F and -10°F (Graph 41) showed that both enzyme systems retained some activity throughout the storage period. Samples stored at 15°F showed a greater loss of activity than those stored at -10°F, and at each temperature, samples stored in an atmosphere of oxygen showed greater loss of activity than those stored under nitrogen. It was also observed that bleaching activity decreased at a faster rate than lipoxygenase activity, particularly during the early storage period. The rate of loss of bleaching activity decreased with increasing storage time, while lipoxygenase activity decreased at a more constant rate during storage. Loss of lipoxygenase activity during storage was also found by Rhee and Watts (1966b). The times for 50% reduction in activity in samples stored under oxygen at 15°F were approximately 4 months and 10 months for bleaching and lipoxygenase activities respectively. The build-up of peroxides and peroxide-breakdown products during storage, as well as normal freezing denaturation, were probably responsible for the loss of enzyme activities.

The presence of both types of enzyme activities in stored frozen unblanched peas can be considered as further evidence that a bleaching mechanism was probably responsible for loss of total pigment during storage (Table 3.16). Blanched peas and beans showed no evidence of enzyme activity and also considerably less pigment destruction. The high TBA values and peroxide concentrations of unblanched peas is in agreement with such a mechanism, and it is apparent that in unblanched vegetables, pigment oxidation represents one of the mechanisms of colour deterioration.



GRAPH 41

Effect of storage on enzyme activity of frozen unblanched peas.

A Chlorophyll Bleaching
B Lipoxygenase

——— Stored under N_2 at $-10^{\circ}F$
– – – " " O_2 " "
–•–• " " N_2 " $15^{\circ}F$
••••• " " O_2 " "

The absence of bleaching and lipoxygenase activities in frozen blanched peas and beans was expected because of the lower heat stability of lipoxygenase compared to peroxidase (Walker 1964b ; Rhee and Watts 1966b). The small loss of total pigment and the high peroxide values and TBA values in some frozen bean samples, however, cannot be explained in terms of the enzymic bleaching system just discussed, and for these samples it would appear that induced oxidation of lipids, and subsequent decomposition of hydroperoxides, was initiated by the blanching conditions used and/or the presence of other lipid oxidation catalysts.

3.64 Pigments from Frozen Peas and Bleaching Systems

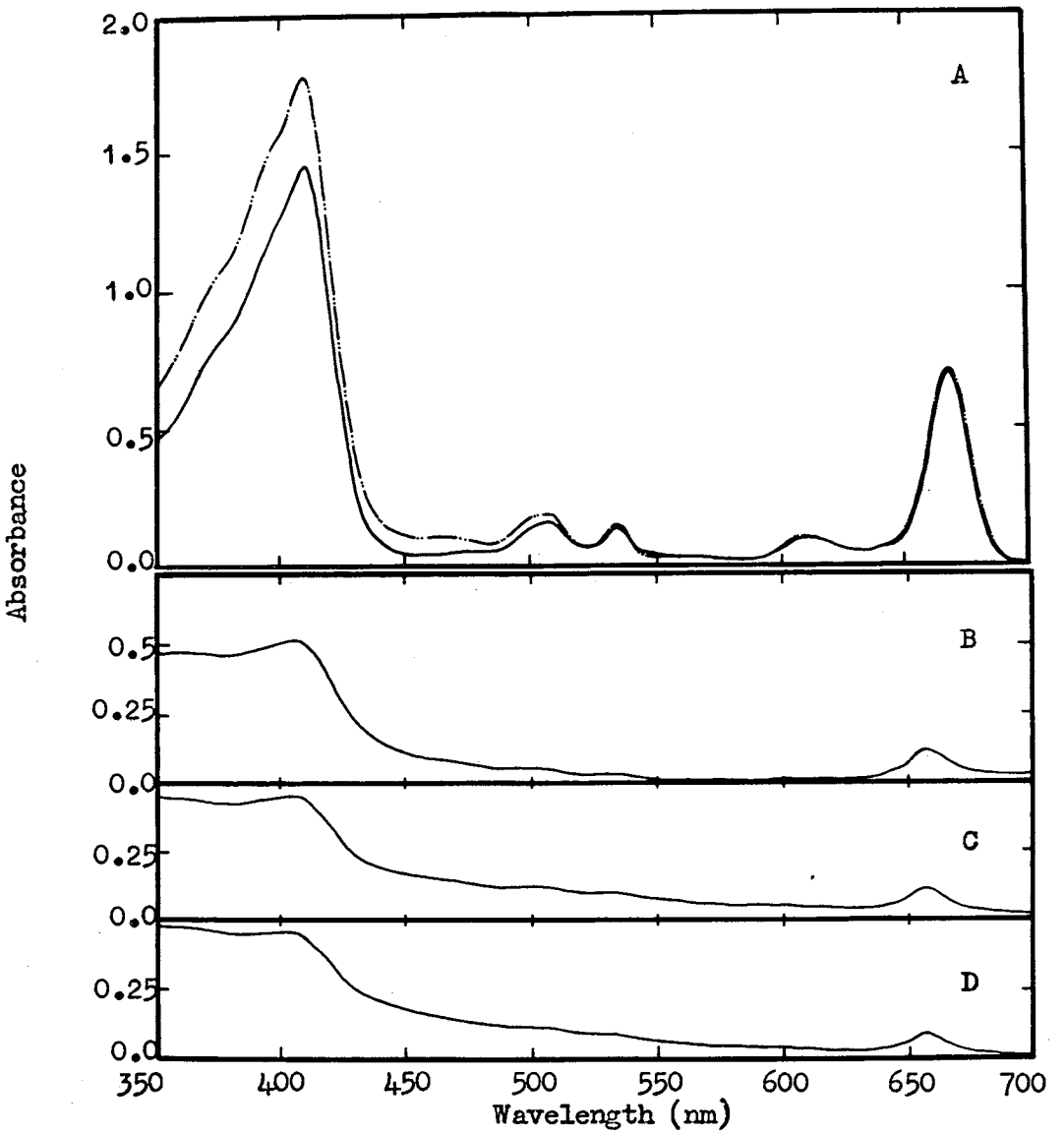
Chromatography of acidified pigment extracts (Expt.35) from unblanched peas stored for 20 months at 15°F (Table 4.92) and blanched peas stored for 20 months at -10°F (Table 4.93) showed no compounds with unusual spectra. Unblanched peas contained considerable quantities of slow-moving pigments (bands F6 and F7) with absorption spectra almost identical to the phytol-free derivatives pheophorbides a and b respectively, in agreement with previous observations (Tables 4.58, 4.59). Blanched peas contained four principal pigments, pheophytins a and b, β-carotene and lutein (after acidification) as well as 2 minor yellow pigments, probably the carotenoids violaxanthin and neoxanthin (Strain 1958).

Curves of bleached crude pigments (Graphs 34,36) indicated that colourless compounds were produced during the course of the reaction, since absorption at all wavelengths decreased and no new peaks appeared. This does not include the peak near 645nm of chlorophyll b which,

although decreasing in size, becomes more prominent due to the more rapid destruction of chlorophyll a (Graph 34). These preliminary observations were in agreement with the findings of Holden (1965a), who could not detect any coloured degradation products of bleached pigments on paper chromatograms.

As the chromatography of bleached pigments has not previously been reported in any detail, it was considered of interest to examine the pigment composition of bleached pure pheophytins a and b. These pigments were used to avoid pheophytin production with Mg-containing compounds and to compare any detectable products with unusual pigments isolated from processed pea puree in Part I of this work. Purified pigments were bleached in a system containing linoleic acid and soya extract for up to 2 hours at room temperature (Expt.36), and chromatographed on thin layers of cellulose. Soya extract was used as the source of the bleaching factor since preliminary examination had showed that it contained no contaminating pigments after bleaching, whereas crude pea enzyme contained small amounts of chlorophyll.

Pheophytin a bleached for 5 minutes (Table 4.94) showed 6 detectable bands, 3 of which (BL3, BL4, BL5) showed almost identical spectra (Graph 42). These compounds were present only in minor quantities compared to band BL2 (Graph 42), but the total pigment concentration had decreased markedly from the unbleached sample (BL1). Band BL2 gave almost identical absorption maxima to unbleached pheophytin a (BL1), but the spectrum showed an increased absorption near 450nm. This effect was more noticeable in the more polar compounds (BL3,BL4) where absorp-



GRAPH 42

Spectral curves of pigments in diethyl ether from bleached pheophytin a

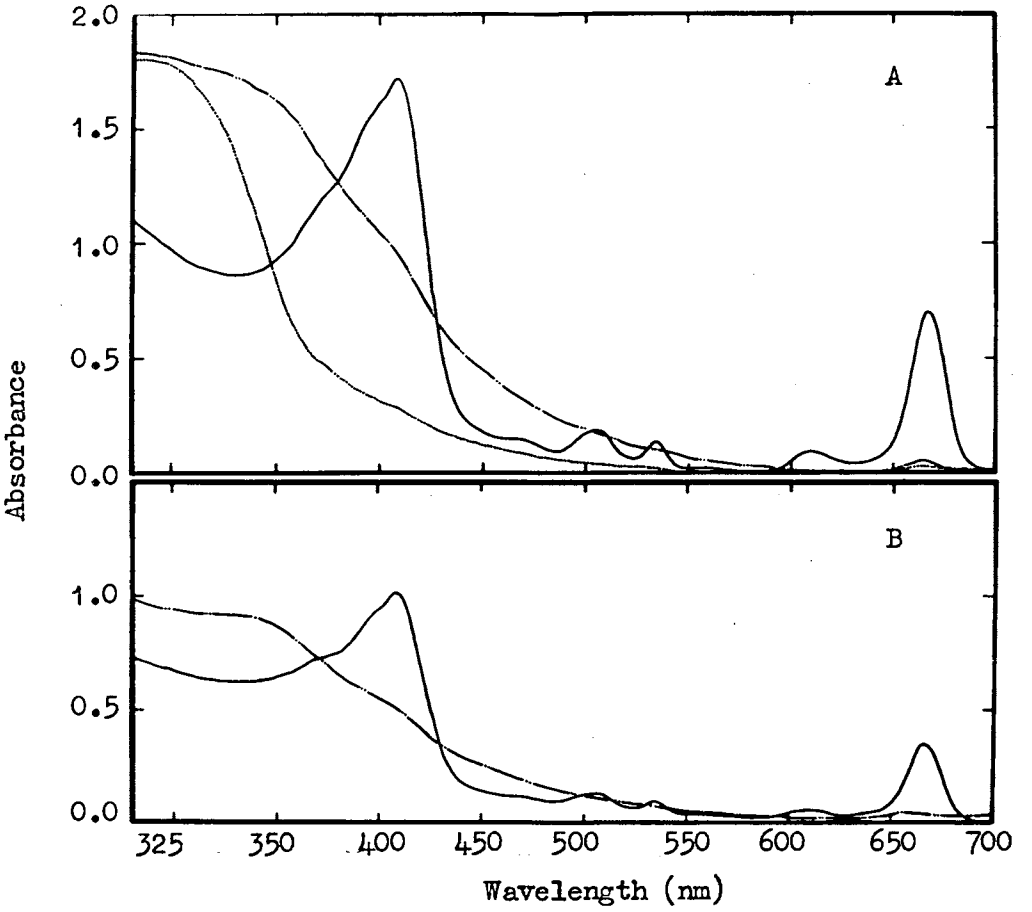
A	———	BL1	pheophytin a
	—•—•—	BL2	
B		BL3	(40mm)
C		BL4	(")
D		BL5	(")

tion towards the blue end of the spectra increased considerably.

Bleaching of pheophytin a for 30 and 120 minutes gave progressively smaller amounts of the principal grey bands BL8 and BL11 (Graph 43.). With longer incubation, the UV absorbance increased, although the absorption maxima in the visible region remained essentially the same. The minor pigments isolated after 5 minutes bleaching were not apparent after extended incubation, and the only detectable bands were yellowish-brown (BL9, BL10, Graph 43), showing one very faint "peak" in the visible region. The slower moving band showed the greater UV absorption.

From these observations it is apparent that the original pheophytin a was progressively converted into slower-moving derivatives with loss of the conjugated double-bond system. Less than 10% of the original pigment remained after 2 hours incubation, and this band, despite the similar absorption maxima, showed substantial "background" absorption towards the UV end of the spectrum. Chromatography of incubated control solutions containing pigment, linoleic acid and heat-inactivated enzyme extract produced only minute amounts of a band with mobility similar to BL7,BL10 and BL12. All other bands, however, were present only after bleaching.

Pheophytin b showed different bleaching behaviour to pheophytin a and formed similar degradation products at each incubation period (Table 4.95). Bleaching for 5 minutes produced 2 minor pigments (BL15, BL16) in addition to unchanged pheophytin b (BL14, Graph 44), and these pigments were also present as degradation products after longer incubation periods. After 30 minutes incubatio, band BL18 (Graph 44) was



GRAPH 43

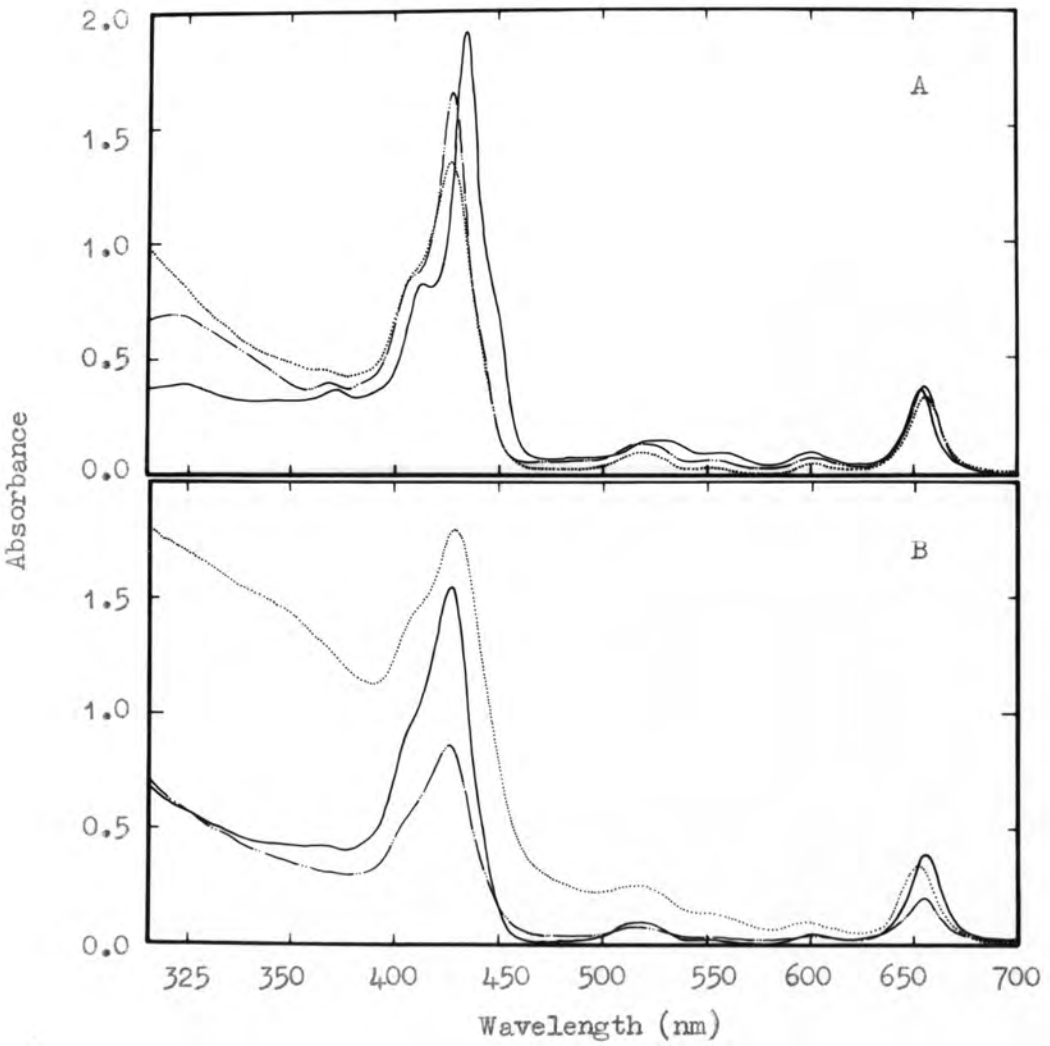
Spectral curves of pigments in diethyl ether from bleached pheophytin a

A	———	BL8	(40mm)
	—•—•	BL9	(")
	•••••	BL10	(")
B	———	BL11	
	—•—•	BL12	(")

present at a concentration only slightly less than unchanged pheophytin b (BL17), but greatly in excess of BL19 (Graph 44). Both of these pigments (BL18, BL19) gave absorption maxima very similar to pigment Y isolated from processed pea puree, although the mobilities and blue/red absorption ratio were different. The slower-moving band (BL19) showed a greater absorption near the UV with a consequent loss of definition in the "blue" band. The UV absorption increased even further in pigments BL20, BL21 and BL22 (Graph 44), similar to that observed with bleached pheophytin a pigments. Bands BL19 to BL22 were present at approximately equal concentrations and all showed fairly similar maxima.

Further incubation showed a decrease in concentration of pheophytin b (BL23) with an increase in concentration of band BL24 (Graph 45), identical to BL15 and BL18. Band BL25 (Graph 45) appeared similar to BL16 and BL19, but the slowest moving band (BL26, Graph 45) showed different maxima to the corresponding band after 30 minutes incubation (BL22, Graph 44), although the shape of the curves were very similar. Approximately 20% pheophytin b appeared unchanged after 120 minutes bleaching, but the coloured degradation products did not account for all of the original total pigment.

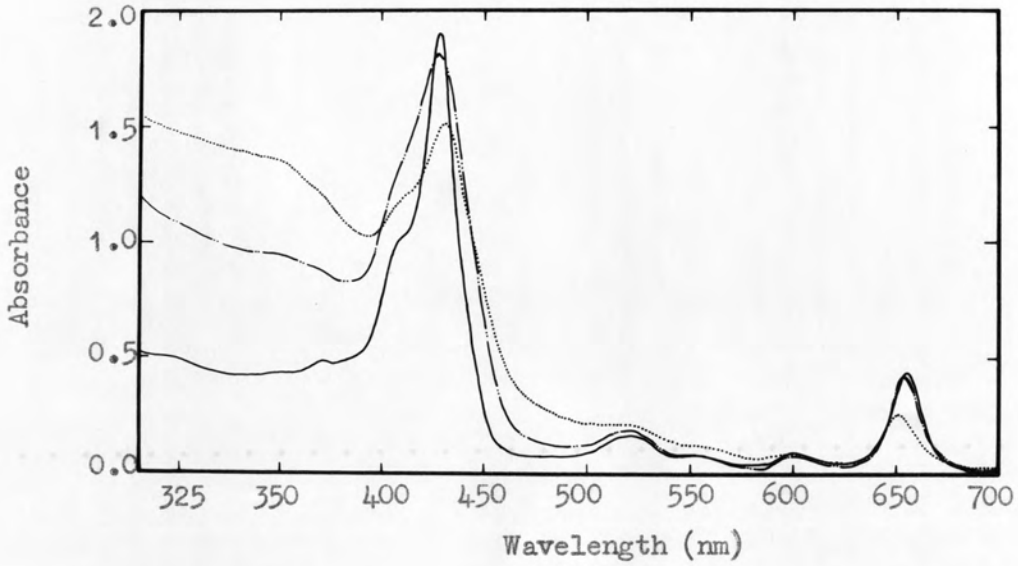
The bleaching of pure pigments in this study was not nearly as rapid as the bleaching of crude solutions observed previously, due to their low solubility in solution. Nevertheless, these results showed that bleaching produced considerable degradation of the pigment molecule, and the rapid loss in colour was indicative of disruption of the con-



GRAPH 44

Spectral curves of pigments in diethyl ether from bleached pheophytin b

A	———	BL14	pheophytin b
	— · · —	BL18	
	· · · · ·	BL19	(40mm)
B	———	BL20	(")
	— · —	BL21	(")
	· · · · ·	BL22	(")



GRAPH 45

Spectral curves of pigments in diethyl ether from bleached pheophytin b

——— BL24
-•·- BL25 (40mm)
····· BL26

jugated double bond system. The failure to observe any of the above degradation products in extracts from frozen unblanched peas is not unexpected since the reactions may have proceeded somewhat differently *in vivo* to those observed in model systems, and resulted in no accumulation of coloured intermediate degradation products. Coloured pigments isolated from bleached pheophytins *a* and *b* did not represent the total pigment lost i.e. not present in the unchanged band, and since only 35% total pigment was lost after 20 months storage, any coloured degradation products would be present in low concentration.

The similarity to pigment Y of pigments from bleached pheophytin *b* is interesting, and shows that this type of reaction (loss of ring V etc.) may represent the first stage in the degradation of pheophytin *b* by a number of mechanisms. Oxidation by free radicals from hydroperoxide decomposition would be expected to attack the cyclopentanone ring also, particularly at the C-10 hydrogen, and it is therefore not surprising that some of the pigment degradation products show properties which would be consistent with such a mechanism.

3.65 Summary of Changes in Stored Frozen Vegetables

It is obvious from the preceding discussion that frozen peas and beans showed greatly different storage behaviour. Relationships between chlorophyll pigments, objective colour, lipid oxidation and pH were found for most samples. With frozen unblanched peas, enzyme activity was probably the most important factor involved, but for the other samples, different mechanisms must be invoked.

Considering firstly frozen peas, blanching had a considerable effect

on the storage stability. In all cases, unblanched peas showed greater chemical changes than blanched peas and these changes could be correlated in some way with residual enzyme activity. For example, unblanched peas showed a low pH consistent with enzymic liberation of free fatty acids which resulted in a high percent conversion of chlorophylls to pheophytins and thus unattractive colours. Residual activity of lipoxygenase and bleaching enzyme systems was consistent with the initial formation of large quantities of hydroperoxides and their subsequent destruction, with the accumulation of TBA-reactive substances in increasing concentration, and with the loss of total chlorophyll-type pigments and carotenoids, resulting in significant changes in Hunterlab colour indices. The presence of lipoxygenase in unblanched peas was consistent with previous reports. Concentrations of TBA-reactive substances indicated that lipid oxidation was not the main cause of off-flavours in frozen raw peas, since relatively low TBA values were found compared to published results on rancid animal products. Nevertheless it must still be considered a possibility that peroxide decomposition in plant foods may proceed differently to animal products, with low concentrations of breakdown products producing undesirable flavours.

Preliminary experiments with soya extracts substantiated the presence of an enzyme system catalysing the destruction of unsaturated fatty acid hydroperoxides with the subsequent bleaching of chlorophyll pigments. The bleaching factor was not lipoxygenase itself, also present in soya extracts, since this enzyme alone, or the hydroperoxides produced from polyunsaturated fatty acids, did not bleach chlorophyll without the

addition of crude extract. Extracts from fresh peas showed the presence of a similar bleaching factor in addition to lipoxygenase activity. Hydroperoxide decomposition was implicated in chlorophyll destruction since (1) absorption at 234nm and hydroperoxide content decreased at high enzyme levels and long incubation periods, consistent with the presence of a bleaching factor in low concentrations in crude extracts; (2) absorption at 280nm due to hydroperoxide decomposition products increased at all enzyme levels and incubation times, but particularly at high enzyme levels and long incubation periods; (3) neither peroxidised linoleic acid nor hemin caused bleaching of chlorophyll, but when added together they destroyed pigment; (4) the addition of hemin to normal or oxidised linoleic acid and pea extract increased bleaching due to accelerated decomposition of hydroperoxides.

The presence of lipoxygenase and chlorophyll bleaching activities in stored frozen unblanched peas thus provides an explanation for some of the changes in pigments, colour, lipid oxidation and pH found in this study. While this mechanism may not account for all changes observed, the prolonged storage stability of blanched peas is in agreement with such a scheme. Changes in stored frozen beans, however, could not be attributed to enzyme systems since these samples were adequately blanched before storage. Compared to frozen blanched peas, frozen beans showed much greater chlorophyll conversion, colour changes, lipid oxidation and pH changes. The much lower initial pH of beans was undoubtedly responsible for some of these changes, but the substantial lipid oxidation was unusual in view of the lower lipid content

of beans. Considering the bean samples studied, correlations between various analyses were of interest. Tendergreen variety beans showed, in general, greater storage stability than the Pearlgreen samples, and within each variety, samples grown at Brisbane were much more susceptible to quality changes than those grown at Bathurst. It was observed that Pearlgreen samples showed a lower initial pH, lower concentrations of total pigment and total chlorophyll, higher TBA values and a small loss of total pigment. The last two findings were significant in view of the relationship between peroxide decomposition and pigment destruction observed with peas, although in the beans a non-enzymic mechanism would have to be involved as suggested by Walker (1964a,b). Additionally, it was observed that both varieties grown at Brisbane showed trends in other variables, including higher percent chlorophyll conversion, higher peroxide values and a greater pH change. While some of these differences were minor, it is of interest that the Brisbane samples were also given a more severe blanching treatment and stored for some time before analyses were performed. From these results it is apparent that the Pearlgreen beans, particularly the samples grown in Brisbane, were much more susceptible to quality changes during storage than were the Tendergreen beans.

Despite their lower lipid content, beans showed greater lipid oxidation than blanched peas, and of the same order as unblanched peas. Since enzymic mechanisms were not involved, at least with regard to lipoxygenase or the bleaching enzyme(s), it would appear that bean lipids were inherently less stable, possibly because of a lack of natural antioxidants, the presence of larger quantities of highly unsaturated fatty

acids (e.g. linoleic acid), or the presence of other lipid oxidation catalysts such as hematin compounds.

In all cases, storage at 15°F resulted in substantially greater changes than storage at -10°F, and the presence of an atmosphere of nitrogen slowed down most reactions. For long-term storage stability, adequate but not excessive blanching, low temperatures and inert atmospheres would appear to be desirable.

4. EXPERIMENTAL

PRELIMINARY EXPERIMENTS

1. Calibration of Spectrophotometers

A Unicam S.P.600 manual spectrophotometer (350nm to 1000nm) and a Unicam S.P.800 double-beam recording spectrophotometer (190nm to 700nm), were checked for accuracy and reproducibility of wavelength and absorbance according to the manufacturer's handbook. Wavelength accuracy was continuously checked throughout the course of the work by measuring or recording the spectrum of a holmium or didymium filter and comparing wavelength tolerances with those reported in standard handbooks. Absorbance accuracy and reproducibility of the Unicam S.P.800 was determined using a solution of potassium dichromate (120 ± 0.5mg/litre) in $^{N}/_{100}$ sulphuric acid according to the method outlined in the instruction manual. Absorbance accuracy for both instruments was also checked using a series of "Chance" neutral glass filters obtained from the National Standards Laboratory, Sydney. The absorbance values of the filters are presented in Table 4.01. In some parts of the work, a Cary 15 recording spectrophotometer was also used. Wavelength and absorbance were calibrated according to the instruction manual.

Table 4.01 Absorbance Values of Neutral Glass Filters Measured by Two Spectrophotometers

Filter Code No.	Wavelength (nm)	Calibrated Absorbance of Filter	Absorbance	
			Unicam S.P. 600	Unicam S.P. 800
NG 159	504	0.0385	0.036	0.040
"	604	0.038	0.0345	0.040
NG 130	504	0.1525	0.154	0.157
"	604	0.1775	0.175	0.180
NG 166	504	0.315	0.316	0.330
"	604	0.356	0.356	0.375
NG 180	504	0.4525	0.468	0.468
"	604	0.501	0.507	0.520
NG 167	504	0.597	0.608	0.630
"	604	0.639	0.650	0.682
NG 100	504	0.7675	0.785	0.820
"	604	0.864	0.895	0.940
NG 174	504	1.022	1.040	1.128
"	604	1.070	1.065	1.180
NG 150	504	1.240	1.270	1.345
"	604	1.475	1.550	1.608

2. Purification of Solvents

Organic solvents used without further purification for pigment work included acetone (A.R.grade, May and Baker); methanol; petroleum ether, boiling fractions 40°-60°C and 60°-80°C; and n-propanol (all A.R.grade, By-products and Chemicals), and were stored at 37°F in the dark. Diethyl ether (anaesthetic grade B.P., D.H.A.) containing 0.002% w/v hydroquinone as preservative, was purified for use in spectrophotometry by distillation and treatment with ferrous sulphate solution. Diethyl ether was twice distilled using a clean glass Quickfit fractionation column, stored over anhydrous calcium chloride at 37°F in the dark in tightly stoppered Winchester bottles and used as soon as possible following distillation. When redistilled ether was left standing for some time, peroxides formed were removed by the method of Vogel (1961) as follows.

About 1 litre ether was shaken twice in a large separatory flask with 10-20ml concentrated ferrous sulphate solution (60g crystallised ferrous sulphate, 6ml concentrated sulphuric acid, and 110ml water) diluted with 100ml water. The aqueous phase was removed, and the ether layer transferred to a Winchester bottle and stored over anhydrous calcium chloride. Before use, the ether was filtered to remove suspended drying agent. Ether prepared by the above procedure was found to give the same spectral readings using pure chlorophyll pigments, as A.R.grade diethyl ether (B.D.H.), and because of its lower cost, was used throughout this work. The presence of peroxides was continually checked by the liberation of iodine from acidified potassium iodide.

3. Column Packing for Chromatography

Dry-packed and wet-packed columns were prepared using sugar, cellulose or sugar-cellulose mixtures, depending on the amount of pigment required and the types of pigments to be separated. The sugar used was commercial "Sunny Cane" powdered icing sugar mixture containing 3% cornflour to prevent caking. The mix was sieved through fine mesh stainless steel screens and used without further treatment. Drying of sugar in ovens or under vacuum was found to give a product that packed poorly and gave uneven flow rates. Mixtures of powdered sugar and cellulose powder (Whatman CF11 column chromedia, ashless cellulose) at concentrations of 125:30w/w have been shown to give ideal columns when prepared by wet-packing techniques. For dry-packed and wet-packed columns in this investigation, 10%w/w cellulose powder in sugar was found satisfactory; separation of the bands was similar, but the solvent flow rate was

increased allowing faster separations to be carried out.

(i) Dry-Packed Columns: Dry-packed columns of sugar or sugar-cellulose mixture were prepared by the following method: Glass tubes of varying size (30 x 1.5cm to 60 x 7cm), were thoroughly cleaned with chromic acid and water, rinsed with petroleum ether and dried with clean compressed air. The smaller tubes had a Quickfit stopcock at the lower end, whereas the larger tubes were slightly constricted before tapering into a B24 Quickfit joint, the constrictions enabling sintered glass discs to rest at right angles to the tube walls. Plugs of glass wool were placed above the stopcock or sintered disc, and held firmly in place by means of a vacuum applied at the lower end. Small amounts of powdered sugar or sugar-cellulose were tamped firmly into place using a stainless steel disc slightly smaller than the inside diameter of the column. Successive additions of adsorbent were packed firmly together, a vacuum being continually applied to the lower end of the column. The upper surface of the adsorbent, 4-5cm from the end of the column, was covered with a disc of filter paper cut to correspond to the tube diameter. Prior to use, the upper part of the column was wet with petroleum ether (b.p.60-80°C), and the solution to be chromatographed was added to the top of the adsorbent, care being taken not to disturb the packed adsorbent surface. This enabled the column, which usually contained some occluded air, to settle properly so that satisfactory pigment bands were formed.

(ii) Wet-Packed Columns: Wet-packed columns of sugar, cellulose (Whatman CC41 cellulose powder for thin-layer chromatography), or sugar/10%w/w cellulose (Whatman CF11 column chromedia) mixtures were prepared

by making a slurry of the adsorbent in petroleum ether (b.p.60-80°C), removing occluded air by vacuum in a stoppered buchner flask, and carefully pouring the slurry into a clean column containing a plug of glass wool. The column was tapped gently with a rubber rod and the adsorbent allowed to settle by running solvent continuously through the column under a slight pressure of dry, oxygen-free nitrogen by means of a pressure-feed reservoir (Edwards 1963). When settled, the upper layer of adsorbent was covered with a disc of filter paper and the pigment solution carefully added. Alternative methods of wet-packing sugar, such as adding dry adsorbent to a column of petroleum ether, were unsatisfactory since occluded air hindered separation of the pigment bands.

4. Purification of Chlorophyll Pigments

4.1 Chlorophyll a

Several methods for separation and purification of chlorophylls were tested for their simplicity and the purity of the prepared pigments. The procedure usually adopted for the preparation of useful quantities of chlorophyll a, described below, was based on reports of Holt and Jacobs (1954), Perkins and Roberts (1962) and Strain et al.(1963).

Fresh market spinach leaves, freed of midribs, were thoroughly washed, and diseased leaves discarded. The leaves were blanched in vigorously boiling water for one minute and immediately cooled in running cold water, excess water being squeezed out by hand. Pigments were extracted from leaves by one of two methods in a room darkened to avoid pigment decomposition. The first involved successive blending of leaves with ice-cold acetone, followed by vacuum filtration of the pulp through

sintered glass. Extraction was repeated until most of the pigments had been removed, the aqueous acetone extract was then extracted with petroleum ether (b.p.60-80°C), and 10%w/v sodium chloride (A.R.grade) added to phase the pigments into the upper layer. The pigment-free aqueous acetone solution was discarded. The second method involved extraction of pigments from shredded leaves using a 1:4 mixture of petroleum ether (b.p.60-80°C) and methanol in a large beaker. The dark green chlorophyll extract was filtered through a plug of cotton wool into a large separatory flask and the leaves re-extracted with the same solvent mixture. After a third extraction, the leaves were almost colourless. The extracts were combined, 10% sodium chloride added to give two phases, and the lower aqueous layer discarded. This latter method appeared the most useful for large quantities of pigments.

Petroleum ether extracts, obtained by either method, were evaporated to about 100ml in a Buchi rotary vacuum evaporator, and scrubbed successively through two 250ml lots of 70% v/v methonal, three lots of 80% v/v methanol and five lots of 10% sodium chloride or saturated magnesium carbonate solution (Lab.Reagent). The scrubbing apparatus was a modification of that described by Mackinney (1940) and consisted of two 500ml B19 Quickfit separatory flasks positioned above each other on a laboratory stand. To the delivery spout of the upper flask was attached, by a B19 socket, a scrubbing tube 23cm long tapering to 2mm internal diameter at the end. The petroleum ether solution was added to the upper flask and the scrubbing solution added to the lower flask. The pigment extract passed through the orifice of the tube and was scrubbed by the washing

solution as it rose to the surface in a fine jet. When phases had separated in the lower flask, the aqueous layer was discarded, the position of the flasks reversed, and the extract scrubbed through a further lot of aqueous methanol or salt solution. The petroleum ether solution, now free of acetone or methanol, was dried over anhydrous granular sodium sulphate (A.R.grade) and decanted into a round-bottom B24 Quickfit flask. The sodium sulphate was washed with small quantities of petroleum ether (b.p.60-80°C) to remove adsorbed pigments, and the extract plus washings vacuum evaporated at room temperature to a small volume, care being taken to avoid precipitation of the pigments from solution.

The pigment solution was adsorbed onto the top of one or more columns packed with powdered icing sugar containing 10%w/w Whatman CF11 powdered cellulose (sugar/cellulose mixture). The column was washed with petroleum ether (b.p.60-80°C) and then with 0.5%v/v n-propanol in petroleum ether (b.p.60-80°C). Upon development with the latter solvent, bands appeared in order of increasing adsorbance as follows:

S1	Yellow	S5	Blue-green
S2	Pale grey	S6	Yellow
S3	Yellow	S7	Olive green
S4	Narrow, pale blue-green	S8	Yellow

When the chromatogram had developed sufficiently, excess solvent was removed by application of a vacuum to the lower end, and the adsorbent pushed out the top of the column.

The blue-green band of chlorophyll a (S5) was cut from the column, transferred to a sintered glass filter and the pigment extracted with acetone. The acetone was evaporated under vacuum at room temperature,

the pigment taken up in petroleum ether (b.p.60-80°C) and added to a second sugar/cellulose column. The column was again developed with n-propanol (0.5%) in petroleum ether (b.p.60-80°C) and the major part of the chlorophyll a band cut out of the dried column. The pigment was further chromatographed as described above, the band cut out, excess solvent removed by filtering on sintered glass and the pigment extracted from the adsorbent by dry purified diethyl ether. A small amount of anhydrous sodium sulphate was added to clarify and dry the solution which was decanted, evaporated to dryness, taken up in ether, and made up to volume at 68°F using 'A' quality calibrated volumetric glassware (E-mil green line). Pigment solutions were stored in the dark at -20°F when not in use.

The visible absorption spectrum of purified chlorophyll a was recorded (Unicam S.P.800) in matched, stoppered 1cm silica cuvettes with diethyl ether as a blank. Absorbances at wavelengths of maximum absorption and at 700nm to correct for turbidity were determined using the Unicam S.P.600 and checked with the Cary 15 spectrophotometer.

Pigment concentrations were determined by evaporating aliquots (usually 25ml) to dryness in a tared calibrated volumetric flask using a stream of dry, oxygen free nitrogen. The flask was placed in a vacuum desiccator over phosphorus pentoxide, and then weighed. Blank determinations were run using diethyl ether only, and pigment concentrations determinedusing the relationship $A_\lambda = \alpha_\lambda cd$

where A_λ = absorbance of solution at wavelength λnm
α_λ = specific absorption coefficient (l/g.cm) at wavelength λnm
c = concentration of pigment (g/l)
d = length of the light path, in this case 1cm.

Spectral data on samples from three of the many isolations of chlorophyll a are presented in Table 4.02. The absorption spectrum is given in Graph 1.

Sample calculation:

A sample from preparation 1, containing 0.00580g/25ml diethyl ether solution, gave an absorbance reading of 0.572 at 660nm when diluted forty times with ether.

From $A_\lambda = \alpha_\lambda cd$

$$\therefore \alpha_{660} = \frac{A_{660}}{cd} = \frac{0.572 \times 40}{0.232}$$

$$\therefore \alpha_{660} = 98.6$$

4.2 Pheophytin a

Known volumes of purified chlorophyll a solution in diethyl ether (Expt.4.1) were acidified by addition of 1 drop concentrated hydrochloric acid (A.R.grade), allowed to stand in the dark for 30 minutes, and scrubbed thoroughly with 5 lots 10% sodium chloride. After drying over anhydrous sodium sulphate the solutions were decanted, made up to the initial volume with dry diethyl ether, and spectral data determined as before. Duplicate samples in which scrubbing was omitted, sodium sulphate being added directly after the addition of hydrochloric acid, were found to give results similar to the scrubbed solutions but sometimes with slightly higher absorbances at 700nm. Spectral data for pheophytin a is presented in Table 4.02. The absorption spectrum is given in Graph 1.

4.3 Chlorophyll b

The large olive green band of chlorophyll b (S7, Expt.4.1) was cut

from the extruded column, the pigment extracted with acetone, vacuum evaporated to dryness, dissolved in petroleum ether (b.p.60-80°C), and chromatographed on a sugar/cellulose column using 1% n-propanol in petroleum ether (b.p.60-80°C) as developer. Small amounts of chlorophyll a or carotenoids were thus separated and the major part of the chlorophyll b band was isolated, the pigment extracted with acetone, evaporated to dryness, taken up in petroleum ether (b.p.60-80°C) and adsorbed onto another column of sugar/cellulose. The column was developed with 1% n-propanol in petroleum ether, the green chlorophyll b dug out of the dried column, eluted with diethyl ether, dried over sodium sulphate, decanted and made up to volume with dry diethyl ether. Spectral data for chlorophyll b are presented in Table 4.02. The absorption spectrum is given in Graph 2.

4.4 Pheophytin b

Purified ether solutions of pheophytin b were prepared from chlorophyll b using the same methods as described for the preparation of pheophytin a from chlorophyll a (Expt.4.2). Spectral data for pheophytin b are presented in Table 4.02. The absorption spectrum is given in Graph 2.

Table 4.02 Spectral Data of Chlorophyll Pigments in Diethyl Ether

Pigment	Preparation No.	Absorption Maxima (nm)	Absorbance Ratio blue/red	Absorption Coefficient (l/g.cm) at Red Maximum
Chlorophyll a	1	410,429,500,533,577,615,660	1.32	98.6*, 100.2*
"	2	410,429,500,532,576,614,660	1.33	102.9, 97.8
"	3	410,428,499,532,577,615,660	1.33	103.0, 100.9 Mean: 100.4

* Duplicate determinations on aliquots from the same solution.

Contd.

Table 4.02 (Contd.)

Pigment	Preparation No.	Absorption Maxima (nm)	Absorbance Ratio blue/red	Absorption Coefficient (l/g.cm) at Red Maximum
Pheophytin a	1	409,468,506,534,561,610,667	2.03	58.4, 56.2
"	2	409,468,506,534,560,609,666.5	2.07	60.2, 57.8
"	3	408,470,506,534,560,609,667	2.08	59.5, 61.6 Mean: 58.95
Chlorophyll b	1	429,453,548,570,594,642	2.84	59.7, 56.1
"	2	428,453,550,568,594,642.5	2.81	57.4, 61.8 Mean: 58.8
Pheophytin b	1	412,433,524,556,599,653	5.16	35.4, 38.6
"	2	412,434,523,555,598,653	5.22	39.1, 37.7 Mean: 37.7

5. Preparation of Chlorophyllides and Pheophorbides

5.1 Crude Chlorophyllide Extract

A crude chlorophyllide extract, containing chlorophyllides a and b, carotenes and xanthophylls, was extracted from spinach leaves by modifications of the method of Schanderl (1962). Fresh spinach leaves, washed thoroughly and freed of midribs, were chopped finely and blended at high speed in an Atomix blender with cold (-10°F) acetone. The final acetone concentration was approximately 66%. The slurry was stored in the dark at room temperature overnight to allow chlorophyllase enzyme action to continue. The slurry was filtered on sintered glass, the pulp washed thoroughly with 80% acetone, and the filtrate scrubbed through two lots of petroleum ether (b.p.60-80°C) to extract unreacted chlorophylls. The acetone solution of chlorophyllides was exhaustively extracted with diethyl ether, the ether solution scrubbed through 10% sodium chloride (adjusted to pH5.5-6.0) and distilled water to remove acetone, and

dried over anhydrous sodium sulphate.

The presence of phytylated pigments was checked by underlayering an aliquot of ether solution with 0.01N potassium hydroxide (A.R.grade). All pigments were extracted into the aqueous solution. The visible absorption spectrum of the ether chlorophyllide solution before and after extraction is shown in Graph 3. The corresponding solutions after conversion by hydrochloric acid are also shown.

5.2 Pheophorbides a and b

Pheophorbides a and b were prepared by acid hydrolysis of purified pheophytin a and pheophytin b solutions using the method of Perkins and Roberts (1962). To one volume of diethyl ether solution of the pheophytin was added 1.6 volumes concentrated hydrochloric acid and the solution allowed to stand at room temperature in the dark for 30 minutes. Following the addition of 2/3 volume of distilled water, the mixture was vigorously shaken four times with one volume of diethyl ether. The acid solution was diluted with 3 volumes of water, 1 volume of diethyl ether added, and the pheophorbides transferred to the ether by vigorous shaking. The ether solutions were washed acid free by scrubbing through several lots of distilled water, dried over anhydrous sodium sulphate, and made to volume. The absorption spectra of pheophorbides a and b are presented in Graph 4. Absorption maxima in diethyl ether are presented in Table 4.03.

Table 4.03 Absorption Maxima of Pheophorbides a and b in Diethyl Ether

Pigment	Absorption Maxima (nm)
Pheophorbide a	409,469,506,534,560,609,666.5
Pheophorbide b	412,453,523,555,598,653

6. Analysis of Pigments in Diethyl Ether

6.1 Accuracy

Aliquots of diluted diethyl ether solutions of purified chlorophyll pigments (Expts. 4 and 5) were mixed in varying proportions in a total volume of 200ml (Table 4.05) and pigment concentrations calculated using the equations reported by White et al.(1963). Absorbances of diethyl ether solutions 1 and 3 measured at 642.5,660 and 700nm, while absorbances of solutions 2 and 4 were measured at 653,666.5 and 700nm (Figure 2.3, Section 2.3222). The equations, given below, express results as μ moles pigment/litre of ether solution.

1. $Py_a t+Po_a t = 20.57\ A_{666.5} - 3.56\ A_{653}$ (ether solution 2)
2. $Py_b t+Po_b t = 31.85\ A_{653} - 9.98\ A_{666.5}$ (ether solution 2)

   where $Py_a t = C_a + Py_a$

   $Po_a t = Cd_a + Po_a$

   $Py_b t = C_b + Py_b$

   $Po_b t = Cd_b + Po_b$
3. $Py_a t = 20.57\ A_{666.5} - 3.56 A_{653}$ (ether solution 4)
4. $Py_b t = 31.85\ A_{653} - 9.98 A_{666.5}$ (ether solution 4)
5. $Po_a t$ = Value Eqn.1 - Value Eqn.3
6. $Po_b t$ = Value Eqn.2 - Value Eqn.4
7. $Cd_a + C_a = 17.33A_{660} + 5.83A_{642.5} - 0.664(Py_a t+Po_a t) - 0.375(Py_b t+Po_b t)$ (ether solution 1)
8. $Cd_b + C_b = 23.32A_{642.5} - 4.086A_{660} + 0.0318(Py_a t+Po_a t) - 0.2005(Py_b t+Po_b t)$ (ether solution 1)
9. $C_a = 17.33A_{660} + 5.83A_{642.5} - 0.664Py_a t - 0.375\ Py_b t$ (ether solution 3)
10. $C_b = 23.32A_{642.5} - 4.086A_{660} + 0.0318Py_a t - 0.2005Py_b t$ (ether solution 3)
11. Cd_a = Value Eqn.7 - Value Eqn.9

12. Cd_b = Value Eqn.8 - Value Eqn.10

13. Py_a = Value Eqn.3 - Value Eqn.9

14. Py_b = Value Eqn.4 - Value Eqn.10

15. Po_a = Value Eqn.5 - Value Eqn.11

16. Po_b = Value Eqn.6 - Value Eqn.12

Pigment molecular weight values, for conversion of concentrations into µg/litre, are given in Table 4.04. Average pigment results of duplicate determinations are given in Table 4.05.

Table 4.04 Molecular Weights of Chlorophylls and Derivatives

Pigment	Molecular Weight
Chlorophyll a	893.5
Chlorophyll b	907.5
Pheophytin a	871.2
Pheophytin b	885.2
Chlorophyllide a	615.5
Chlorophyllide b	629.5
Pheophorbide a	593.2
Pheophorbide b	607.2

Table 4.05 Analysis of Chlorophyll Pigments in Diethyl Ether

Pigment	Pigment Concentration (µg in 200ml Total Volume) Solution No.							
	1	2	3	4	5	6	7	8
Pigment Added								
Chlorophyll a	150	100	100	-	-	-	-	25
Chlorophyll b	50	100	50	25	-	-	-	25
Pheophytin a	-	-	25	150	150	50	-	50
Pheophytin b	-	-	25	25	50	50	-	25
Pheophorbide a	-	-	-	-	-	50	150	50
Pheophorbide b	-	-	-	-	-	50	50	25
Total	200	200	200	200	200	200	200	200
Pigment Calculated								
Chlorophyll a	154.0	92.2	93.1	-	-	-	-	23.3
Chlorophyll b	48.7	106.6	55.3	30.1	-	-	-	27.0
Pheophytin a	-	-	24.3	159.1	150.8	49.6	-	47.2
Pheophytin b	-	-	26.2	22.7	44.1	56.4	-	26.7
Pheophorbide a	-	-	-	-	-	48.3	144.3	43.1
Pheophorbide b	-	-	-	-	-	55.4	46.2	22.4
Total	202.7	198.8	198.9	211.9	194.9	207.7	190.5	187.7

6.2 Reproducibility

The reproducibility of extraction and pigment estimation was examined by extracting pigments with acetone from replicate 20.0g samples pea puree prepared by blending commercially canned peas and brine. The pigments were phased into ether, the ether solutions scrubbed thoroughly with 10% sodium chloride (pH5.5-6.0) and distilled water, made to volume (200ml) and absorbances measured at 666.5nm and 700nm. Results are given in Table 4.06.

Table 4.06 Reproducibility of Pigment Extraction and Estimation

Replicate Extraction	Corrected Absorbance at 666.5nm
1	0.409
2	0.407
3	0.417
4	0.414
5	0.406
6	0.410

Efficiency of extraction of chlorophyllides and pheophorbides with dilute alkali was examined by adding 5ml crude chlorophyllide extract in ether (Expt.5.1) to replicate 25ml aliquots of pigment extract 1 (Table 4.06). The ether solutions were extracted three times with 50ml 0.01N KOH, washed repeatedly with 10% sodium chloride (pH5.5-6.0) and distilled water, dried over anhydrous sodium sulphate, the solutions made up to volume (25ml), and absorbances measured at 666.5nm and 700nm. Results are presented in Table 4.07.

Table 4.07 Efficiency of Extraction of Added Chlorophyllides from Ether Solution

Solution	Corrected Absorbance at 666-667nm
1 (initial)	0.409
1a (extracted)	0.401
1b (")	0.395
1c (")	0.413
1d (")	0.407
1e (")	0.400

7. Analysis of Pigments in 80% Acetone

7.1 Wavelengths of Maximum Absorption

Solutions of purified chlorophyll pigments in ether, prepared in

previous experiments, were vacuum evaporated at room temperature and dissolved in 80% acetone. Wavelengths of maximum absorption in the "red" region of the visible spectrum for this solvent are presented in Table 4.08. Also recorded is one of the secondary maxima for pheophytin a.

Table 4.08 Absorption Maxima of Chlorophyll Pigments in 80% Acetone

Pigment	Absorption Maxima (nm)
Chlorophyll a	662
Chlorophyll b	644 - 645
Pheophytin a	665 ; 536
Pheophytin b	654 - 655

The complete equations for pigment estimation by the methods of Dietrich (1958) and Vernon (1960) are given below.

7.2 Method of Dietrich (1958)

$$\text{Percent chlorophyll conversion} = \frac{R_x - R_o}{R_{100} - R_o} \times 100$$

where R_x = absorbance ratio $\frac{A_{534}}{A_{556}}$ for the unknown extract.

R_o = absorbance ratio $\frac{A_{534}}{A_{556}}$ for zero conversion.

R_{100} = absorbance ratio $\frac{A_{534}}{A_{550}}$ for total conversion.

7.3 Method of Vernon (1960)

Before Conversion

1. Chlorophyll a (mg/litre) $= 25.38 \Delta^* A_{662} + 3.64 \Delta A_{645}$

   Total chlorophyll a with no conversion (mg/litre) $= 20.65 A_{666} - 6.02 A_{655}$

2. Chlorophyll b (mg/litre) $= 30.38 \Delta A_{645} - 6.58 \Delta A_{662}$

   Total chlorophyll b with no conversion (mg/litre) $= 32.74 A_{655} - 13.75 A_{666}$

3. Total chlorophyll (mg/litre) = $18.80 \Delta A_{662} + 34.02 \Delta A_{645}$

   Total chlorophyll with no conversion (mg/litre) = $6.90\ A_{666} + 26.72\ A_{655}$

4. Total chlorophyll a with no conversion (mg/litre) = $22.31\ A_{666} - 17.90\ A_{536}$

5. Total chlorophyll b with no conversion (mg/litre) = $97.40\ A_{536} - 22.6\ A_{666}$

6. Total chlorophyll with no conversion (mg/litre) = $79.5\ A_{536} - 0.29\ A_{666}$

7.)
8.) Equations to calculate chlorophylls a and b and total chloro-
9.) phyll only when there is no conversion of chlorophyll to pheophytin. Not used in this study.

After Conversion

10. Pheophytin a (mg/litre) = $20.15\ A_{666} - 5.87\ A_{655}$

10a. Pheophytin a (mg/litre) = $21.67\ A_{666} - 17.42\ A_{536}$

11. Pheophytin b (mg/litre) = $31.90\ A_{655} - 13.40\ A_{666}$

11a. Pheophytin b (mg/litre) = $95.00\ A_{536} - 22.0\ A_{666}$

12. Total Pheophytin (mg/litre) = $6.75\ A_{666} - 26.03\ A_{655}$

12a. Total Pheophytin (mg/litre) = $77.58\ A_{536} - 0.33\ A_{666}$

* Where $\Delta A662$ = change in absorbance at 662nm etc.

PART I CHLOROPHYLLS AND COLOUR IN PROCESSED PEA PUREE

A. STORAGE STUDIES ON H.T.S.T. PROCESSED PEA PUREE

8. Preparation and Processing of Pea Puree

8.1 Raw Material

One hundred and fifty pounds (150 lb.) freshly hand-picked green peas (Pisum sativum, variety Edgell Freezer) grown at Sassafras, Tasmania, were packed in hessian bags and air-freighted at room temperature to Sydney, transported to the Food Technology laboratories at Kensington, and immediately placed in cold storage at 37°F. On the following day, the peas were shelled by hand, damaged and unsuitable material was discarded, and the peas again stored at 37°F until blanched and pureed for heat processing or until prepared for frozen pea work (Part II, Expt.19). The total weight of shelled peas was 58 pounds, representing a 38.7% yield. The maturometer readings determined immediately after picking are given in Table 4.09.

Table 4.09 Maturity Index of Fresh Peas

Test No.	Maturometer Readings	Mean
1	200, 220, 210, 220, 210 220, 190, 210, 210, 220	211
2	220, 220, 240, 260, 200 210, 220, 250, 210, 210	221

8.2 Preparation

An initial experiment was carried out to determine the optimum blanching time for inactivation of the enzyme peroxidase. Duplicate 10g lots of peas were blanched in boiling water for 30,45,60,75,90 and 120 seconds, immediately cooled in cold running water and macerated with 30ml

distilled water as described by Masure and Campbell (1944). 2ml lots of filtered extract were added to 20ml distilled water in a test tube, and 1ml 0.5% guaiacol (in 50% ethanol) and 1ml 0.08% hydrogen peroxide added without mixing. The tube contents were mixed and the development of a brown colour noted. No colour development in $3\frac{1}{2}$ minutes signified a negative test, and hence adequate blanching. The results are given in Table 4.10. Blank tubes were prepared containing pea filtrate but no hydrogen peroxide or guaiacol for each blanching time. Subsequently all peas were blanched for 1 minute in boiling water and immediately cooled in running water.

Table 4.10 Effect of Blanching Time on Peroxidase Activity

Time (Seconds)	Colour Development in $3\frac{1}{2}$ Minutes
0	+++ dark red - brown
30	+++ dark red - brown
45	+ slight red - brown
60	- no colour
75	- no colour
90	- no colour
120	- no colour

Preliminary experiments had indicated that pea puree of a consistency similar to that obtained when canned peas were blended with the canning brine would be suitable for H.T.S.T. processing in thermal death time tubes. Consequently, puree of moisture content 86.0-86.5% was prepared.

Normal pH puree: 440g blanched peas, drained for two minutes after cooling in running cold water, were blended with 240ml distilled water

in an atmosphere of nitrogen in a blender (Kenwood "Chef" Model A.700 Electric food mixer) until a uniform mixture was obtained. The blended mix was then finely comminuted in an Ultra-turrax disintegrator (Model T.45/6) in a tall plastic jar flushed with nitrogen. The average moisture content of puree prepared from 10 pounds freshpeas was 86.3% and the pH 6.95.

Elevated pH puree: 440g blanched peas were blended with 275ml distilled water and 20g magnesium carbonate powder (Laboratory Reagent) in the Kenwood blender, followed by comminution in the Ultra-turrax. The average moisture content was 86.2%, and the pH8.45.

The normal and elevated pH purees each prepared from 10 pounds fresh peas were stored at 37°F under nitrogen in air-tight, half-gallon glass jars until processed.

8.3 Processing and Storage

Before processing, the pea puree was deaerated to remove air incorporated during blending and comminution which was necessary to avoid excessive pressure in the tubes during processing. The apparatus used for deaeration consisted of a 2 litre Quickfit round bottom reaction flask to which was attached a reservoir and a delivery tube leading to an acetone-dry ice bath, vacuum regulator and mechanical vacuum pump. A vacuum of approximately 25 inches mercury was maintained. The puree was sucked through the stop-cock from the reservoir, and was vibrated under vacuum in the reaction vessel for about 10 minutes to remove air bubbles. The vacuum was broken with a slow bleed of nitrogen fed through a three-way tap.

Thermal death time (T.D.T.) tubes used for processing were Pyrex glass, 25cm long x 0.7cm internal diameter and walls 1mm thick (Crown Crystal Glass, Botany, Sydney). The deaerated puree was filled into tubes, sealed at one end, by means of a plastic syringe (8cm x 4cm), to the spout of which was cemented, by inert epoxy resin, a stainless steel tube 27cm long x 0.5cm diameter. This tube was placed inside the thermal death time tube so that no air was incorporated during filling. Approximately 7.5-8.0g puree was filled into each tube, 10 tubes being required for each set of analyses.

The Pyrex T.D.T. tubes were sealed approximately 3cm above the level of the puree by heating the tube in a flame burning equal parts of oxygen from a cylinder and coal gas from the gas mains. The molten glass was drawn out with a pair of pliers to form a seal, using slight rotation of the tube during this operation to give satisfactory results.

Before the puree was processed under H.T.S.T. conditions, process times were determined to give a constant F_o value of 6.0 at 240°F, 260°F, 280°F and 300°F. This involved (1) determination of the rate of heat penetration into T.D.T. tubes of pea puree at each of the process temperatures; (2) calculation, by the General Method of Bigelow et al.(1920), of process times for each process temperature to give $F_o = 6.0$.

Heat penetration rates were recorded by measuring the temperature of puree at the centre of T.D.T. tubes by means of copper-constantan thermocouples. The thermocouple, formed from thin gauge, calibrated wires (Leeds and Northrup Co., Philadelphia, U.S.A.) soldered together at one end only, was held in place in the centre of a T.D.T. tube (open

at both ends) about 2.0cm from one end by means of a plug of "Plastibond" plastic putty (N.P. Croft Aust. Pty. Ltd.). A small constriction was first made around this end of the tube, to ensure that the resin plus was not ejected during processing. The other ends of the copper and constantan wires were attached to terminals on a manual potentionmeter reading directly in ^{o}F (range $-100^{o}F$ to $400^{o}F$, Brown Instrument Division, Minneapolis - Honeywell, Philadelphia, U.S.A.).

T.D.T. tubes were filled and sealed as previously described, and then placed for 15 minutes in a water bath controlled at $100^{o}F \pm 1F^{o}$ by means of a thermostat (Haake, West Germany). The tubes were heated in a bath (30x30x30cm) of glycerine heated to process temperature by an electric immersion heater. This was connected, through a cut-out relay, to a thermometer ($-10^{o}C$ to $150^{o}C$) which thermostatically controlled the bath temperature. The glycerine was stirred continuously and the bath temperature recorded by a copper-constantan thermocouple attached to a 6-point recorder (Honeywell Brown Ltd., England) calibrated for $-50^{o}F$ to $300^{o}F$. The accuracy of the thermocouples was checked with standard mercury-in-glass thermometers complying with British Standard Specification No. 1365.

When the tubes of puree had attained a constant initial temperature ($100^{o}F$), they were immersed in a wire rack into the oil bath and temperatures recorded manually at short time intervals. When the temperature of the puree had reached the bath temperature, the tubes were removed from the oil bath and immediately placed in ice for two minutes. For each process temperature/pH variable (i.e. 8 lots), 10 tubes were processed separately and heat penetration recorded (Table 4.11). No significant

difference was found in heat penetration rate for normal and elevated pH puree at any of the four processing temperatures.

Table 4.11 Average Heat Penetration Data for Pea Puree Processed at 240°, 260°, 280° and 300°F in T.D.T. Tubes

Process Temperature (°F)							
240		260		280		300	
Time (Secs.)	Puree Temp. (°F)	Time (Secs.)	Puree Temp. (°F)	Time (Secs.)	Puree Temp. (°F)	Time (Secs.)	Puree Temp. (°F)
Heating		Heating		Heating		Heating	
0	100	0	100	0	100	0	100
38	181	17	136	30	196	8	122
49	215	31	183	41	218	17	161
60	223	44	213	51	236	28	204
75	230	60	232	63	253	39	236
92	234	75	244	75	262	50	256
103	236	95	252	85	268	66	275
111	237	115	256	96	271	80	285
123	238	135	259	108	275	92	290
132	239	165	260	120	276	100	292
165	240	Cooling		128	277	109	294
Cooling		0	260	140	278	118	296
0	240	22	148	150	279	128	297
15	203			165	280	142	298
				Cooling		152	299
				0	280	165	300
				11	226	Cooling	
						0	300
						11	260
						20	204

The evaluation of process times necessary to give a process value of F_o = 6.0 for pea puree at each of the process temperatures was calculated graphically by the method of Bigelow et al.(1920). From the heat penetration data, lethal values were tabulated for each temperature using data for Clostridium botulinum spores (N.C.A. 1954) assuming Z = 18°F and lethal value = 1.000 at 250°F. The lethal values used in these calculations are presented in Table 4.12. Data for temperatures above 260°F

were obtained from Graph 5 drawn by extrapolation of data given in the above reference (N.C.A. 1954). Results showing lethal value and temperature are given in Graphs 6, 7, 8 and 9.

Table 4.12 Lethal Values for Cl. Botulinum Spores (Z=18)

(where Lethal Value = $10^{-\left(\frac{250-t}{18}\right)}$)

Temperature (^{o}F)	Lethal Value	Temperature (^{o}F)	Lethal Value	Temperature (^{o}F)	Lethal Value	Temperature (^{o}F)	Lethal Value
200	0.002	232	0.100	249	0.880	265	6.76
202	0.002	233	0.114	250	1.000	266	7.69
204	0.003	234	0.129	251	1.14	268	10.00
206	0.004	235	0.147	252	1.30	270	13.16
208	0.005	236	0.167	253	1.47	271	15.38
210	0.006	237	0.190	254	1.67	272	16.94
212	0.008	238	0.215	255	1.90	272.75	18.87
214	0.010	239	0.246	256	2.16	273	19.23
216	0.013	240	0.278	257	2.45	273.25	19.80
218	0.017	241	0.316	258	2.79	273.5	20.41
220	0.022	242	0.359	259	3.16	274	21.75
222	0.028	243	0.408	260	3.60	275	25.00
224	0.036	244	0.464	261	4.13	276	27.77
226	0.046	245	0.527	262	4.69	277	31.75
228	0.060	246	0.600	263	5.26	278	35.72
230	0.077	247	0.680	264	5.92	280	46.73
231	0.088	248	0.774				

Calculation of Process Times to Give F_o = 6.0

(i) 240^{o}F (Graph 6): The total area under the heating and cooling parts of the lethal value curve is 684 units (where 1 square = 100 units and represents F_o = 0.05). For a process value F_o = 6.0, the total area required is 6.0 x 2000 = 12,000 units where 2000 units represents the area of unit lethality (where F_o = 1). Hence the additional area required for F_o = 6.0 is 12,000 - 684 = 11,316 units. Since 1 minute at 240^{o}F is equivalent to 556 units of area, additional process time required = $\frac{11,316}{556}$ = 20.35 minutes = 20 minutes 21 seconds. Hence total process time for F_o = 6.0 at 240^{o}F = 20 minutes 21 seconds + 2 minutes 45 seconds =

23 minutes 6 seconds.

(ii) 260°F (Graph 7): Total area under lethal value curve = 668 units (where 1 square = 100 units and represents F_o = 0.5). For process value F_o = 6.0, area required = 1200 units, hence additional area required = 1200 - 668 = 532 units. Since 1 minute at 260°F is equivalent to 360 units of area, additional process time required = $\frac{532}{360}$ = 1.48 minutes = 1 minute 29 seconds. Hence total process time for F_o = 6.0 at 260°F = 1 minute 29 seconds + 2 minutes 45 seconds = 4 minutes 14 seconds.

(iii) 280°F (Graph 8): Process time required for F_o = 6.0 at 280°F was found to be 1 minute 40 seconds. The maximum temperature in the centre of the T.D.T. tubes was 273°F.

(iv) 300°F (Graph 9): Process time required for F_o = 6.0 at 300°F was found to be 1 minute 8 seconds. The maximum temperature in the centre of the T.D.T. tubes was 277°F.

The T.D.T. tubes, initially at 100°F, were processed in batches of 25 tubes at each process temperature for the times given above, and immediately placed in an ice bath for two minutes.

The tubes for each process temperature/pH combination were mixed together and stored in the dark at 68°F (air-conditioned room), 37°F (cool-room) and -10°F (freezer). The total number of tubes prepared at each of the process temperatures for storage is given in Table 4.13. Ten tubes were required for analysis of pigments, colour, etc. for all stored samples.

Table 4.13 Tubes of H.T.S.T. Processed Pea Puree Prepared for Analysis

Puree pH	Process Temperature (°F)	Storage Time (Months) 0[a]	1[b]	2[c]	3[d]	6[e]	12[e]	18[e]	Total Number of Tubes
6.95	240	10	10	10	20	30	30	30	140
"	260	10	10	10	20	30	30	30	140
"	280	10	10	10	20	30	30	30	140
"	300	10	10	10	20	30	30	30	140
8.45	240	10	10	10	20	30	30	30	140
"	260	10	10	10	20	30	30	30	140
"	280	10	10	10	20	30	30	30	140
"	300	10	10	10	20	30	30	30	140
									1120

a Stored at -10°F until analysed
b Analyses not done
c Analysed after storage at 68°F
d Analysed after storage at 68°F, 37°F
e Analysed after storage at 68°F, 37°F, -10°F

9. Methods of Pigment Analyses of H.T.S.T. Processed Pea Puree

For each group of analyses on any sample of stored puree, 10 T.D.T. tubes randomly chosen were removed from storage and puree extracted from the tubes. Frozen samples were first thawed. The puree content of 10 tubes (75-80g) was thoroughly mixed, and objective colour measurements recorded in duplicate using the Hunterlab Colour and Colour-Difference Meter. Duplicate 20.0g samples puree were weighed for pigment determinations, and duplicate 5g lots weighed accurately to check moisture content by the A.O.A.C. (1960) method. About 5-10g puree was used for pH determination, and the remainder used for subjective colour estimations.

9.1 Extraction

All extractions and other pigment work were carried out in a room darkened to avoid degradative pigment changes. Pigment solutions in

flasks were kept in closed cupboards or covered with black polythene bags.

20.0g samples puree were blended for 3 minutes at high speed with 80ml cold (-10°F) acetone and 3ml distilled water in a stainless steel Atomix blender (M.S.E.). The final acetone concentration was 80%, allowing for water in the puree. About 1g filter aid (Hyflo Super-cel, Johns-Manville) was added and the mixture vacuum filtered through a sintered glass funnel (7cm diameter, porosity 4). The pulp was washed with a little 80% aqueous acetone on the filter and reblended for 3 minutes with 100ml 80% acetone, filtered and washed with a little 80% acetone to ensure all pigments were removed. The filtered solution was transferred to a 250ml volumetric flask equilibrated at 68°F, and made up to volume with 80% acetone. If the filtrate was cloudy before the final addition of 80% acetone, a small volume of pure acetone was added to clarify and then made to volume with 80% acetone. This solution was designated acetone soln. (1).

9.2 Analysis in 80% Acetone

(i) Method of Dietrich (1958): Two 50ml volumetric flasks were taken, to one was added 1.5ml 80% acetone, to the other 1.5ml saturated oxalic acid (A.R.grade) in 80% acetone. Both flasks were made to volume with acetone solution (1) and the flasks kept in the dark at 68°F for about 2 hours. These solutions were designated acetone solutions (2) and (3) respectively. After storage in the dark, absorbance readings were taken at 534,556 and 700nm for both solutions in 1cm stoppered,matched silica glass cuvettes by means of the Unicam S.P.600. Spectra between 350nm and 700nm were recorded using the Unicam S.P.800. In both cases, blank solutions of 80% acetone were used. The absorbance reading at 700nm was

taken as a check on the optical clarity of the solutions. For initial solutions, this reading was usually 0.002-0.005 absorbance units, but occasionally, the converted solution had a higher absorbance at 700nm. In this case, all absorbances of the converted solution were corrected to give the same absorbance at 700nm as the initial pigment extract (acetone solution 2). Calculation of percent conversion of chlorophylls to pheophytins was made using the equation given in Section 7.2.

(ii) Method of Vernon (1960): The initial and converted pigment extracts used to determine percent conversion by the method of Dietrich (1958), i.e. acetone solutions (2) and (3), were also used for pigment determinations by the method of Vernon (1960). The absorbances of both initial and converted solutions, after standing in the dark for 2-3 hours, were also read at536,645,655,662,665,666 and 667nm. Pigment concentrations were calculated using the equations given in Section 7.3.

9.3 Analysis in Diethyl Ether

The method of White et al.(1963) as modified below was used for the estimation of pigments in processed pea puree. 100ml 80% acetone solution (1) was gently mixed with about 50ml purified diethyl ether and 10%w/v sodium chloride solution (pH5.5-6.0) was slowly added to phase the pigments into the upper ether layer. After standing, the lower aqueous acetone phase was run off and extracted with 20ml ether to remove residual pigment. The aqueous solution was discarded, and the two ether extracts combined in a 500ml, B19 Quickfit separatory flask. The ether solution was successively scrubbed free of acetone through five 250ml lots of 10% sodium chloride using the apparatus described previously (Expt.4). The

flasks and scrubbing tube were washed with a little ether after each scrubbing to extract residual pigment remaining on the glass. The ether solution was run off into a 150ml conical flask, and dried over anhydrous sodium sulphate. The dry pigment solution, which was brilliantly clear, was decanted into a 100ml volumetric flask, and the sodium sulphate extracted with small volumes of dry ether to remove adhering pigment. The solution was equilibrated at 68°F in a water bath, and made up to volume with dry ether. This solution was designated ether solution (1). Absorbances were measured at 642.5,660 and 700nm using the Unicam S.P.600.

To a 25ml aliquot of ether solution (1) was added a drop of concentrated hydrochloric acid, the solution stood at room temperature for 2 hours, then washed 5 times with 100ml 10% sodium chloride, dried over anhydrous sodium sulphate, decanted and made up to 25ml volume with dry ether. Absorbances of this solution, designated ether solution (2), were measured at 653,666.5 and 700nm.

A 50ml aliquot of ether solution (1) was also extracted three times with 100ml portions of 0.01N potassium hydroxide to remove phytol-free pigments. The aqueous extracts were discarded. The ether solution was thoroughly washed with five 100ml lots of 10% sodium chloride, dried over anhydrous sodium sulphate and made up to 50ml with dry ether. Absorbances of this solution, ether solution (3), were measured at 642.5,660 and 700nm.

A 25ml aliquot of ether solution (3) was converted by addition of 1 drop concentrated hydrochloric acid, and left in the dark for 2 hours. The solution was then washed five times with 100ml 10% sodium chloride, dried over anhydrous sodium sulphate, and made up to 25ml with dry ether

(ether solution 4). Absorbances were measured at 653,666.5 and 700nm.

Pigment concentrations were determined using the equations described previously (Expt.6). The scheme used for estimation of pigments in pea puree is summarised in Figure 3.1 (Discussion).

10. Pigments of Pea Puree

Results of storage trials on normal and elevated pH pea puree processed under H.T.S.T. conditions are presented in Tables 4.14 to 4.25. Duplicate results by the methods of Vernon (1960) and White et al.(1963) were averaged for each puree analysis. Using Vernon's method, chlorophyll a concentrations were averages of results from equations 1 and 4, chlorophyll b concentrations were averages of results from equations 2 and 5, and total chlorophyll concentrations were averages of results from equations 3 and 6. Total a, total b and total a + b pigments were obtained from equations 10 and 10a, 11 and 11a and 12 and 12a respectively.

Percent conversion figures were derived from the individual pigment concentrations obtained by the methods of Vernon (1960) and White et al. (1963). Percent conversion of total chlorophyll was also checked by the method of Dietrich (1958). Average figures for allmethods are presented. No phytyl-free pigments (chlorophyllides or pheophorbides) were found in any samples of heat-processed pea puree.

Table 4.14 Pigment Content of Pea Puree Processed at 240°F and Stored 18 Months at 68°F

Pigment	Pigment Content (μg/g)													
	Normal pH							Elevated pH						
	Unprocessed	Storage Time (Months) 0	2	3	6	12	18	Unprocessed	Storage Time (Months) 0	2	3	6	12	18
C_a	60.8	5.5	0.0	0.0	0.0	0.0	0.0	54.6	35.0	23.5	20.9	14.6	17.4	22.6
C_b	25.2	10.0	1.2	0.0	0.0	0.0	0.0	22.6	16.4	14.5	11.8	13.7	14.1	14.7
Py_a	3.6	61.1	63.7	64.1	66.4	61.0	63.2	3.2	21.9	31.9	34.8	43.1	30.0	21.4
Py_b	0.0	15.5	22.8	24.6	27.2	26.7	23.6	0.0	5.6	8.7	10.6	10.8	14.9	19.4
Total C	86.0	15.5	1.2	0.0	0.0	0.0	0.0	77.2	51.4	38.0	32.7	28.3	31.5	37.3
Total Py	3.6	76.6	86.5	88.7	93.6	87.7	86.8	3.2	27.5	40.6	45.4	53.9	44.9	40.8
Total C + Py	89.6	92.1	87.7	88.7	93.6	87.7	86.8	80.4	78.9	78.6	78.1	82.2	76.4	78.1
% Conv.C→Py	4.0	83.2	98.6	100	100	100	100	4.0	34.9	51.7	58.1	65.6	58.8	52.3
% Conv.C→Py_a	5.6	92.7	100	100	100	100	100	5.5	39.5	57.6	62.5	74.4	63.3	48.6
% Conv.C_b^a→Py_b^a	0.0	60.8	95.0	100	100	100	100	0.0	25.5	37.5	47.3	44.1	51.4	56.9

Table 4.15 Pigment Content of Pea Puree Processed at 260°F and Stored for 18 Months at 68°F

Pigment	Pigment Content (μg/g)													
	Normal pH							Elevated pH						
	Unprocessed	Storage Time (Months) 0	2	3	6	12	18	Unprocessed	Storage Time (Months) 0	2	3	6	12	18
C_a	60.8	38.3	1.3	2.5	0.0	0.0	0.0	54.6	48.0	37.5	32.7	23.2	20.0	25.8
C_b	25.2	23.5	6.4	3.0	0.0	0.0	0.0	22.6	19.3	20.8	16.6	17.4	17.9	16.5
Py_a	3.6	24.7	62.8	63.9	63.5	60.8	63.0	3.2	10.2	22.1	22.1	32.4	30.8	21.4
Py_b	0.0	2.0	18.5	23.7	23.6	24.3	26.1	0.0	2.4	3.0	5.0	6.9	12.4	16.7
Total C	86.0	61.8	7.7	5.5	0.0	0.0	0.0	77.2	67.3	58.3	49.3	40.6	37.9	42.3
Total Py	3.6	26.7	81.3	87.6	87.1	85.1	89.1	3.2	12.6	25.1	27.1	39.3	43.2	38.1
Total C + Py	89.6	88.7	89.0	93.1	87.1	85.1	89.2	80.4	79.9	83.4	76.4	79.9	81.1	80.4
% Conv.C→Py	4.0	30.1	91.3	94.1	100	100	100	4.0	15.8	30.1	35.5	49.2	53.3	47.4
% Conv.C→Py_a	5.6	39.2	98.0	96.2	100	100	100	5.5	17.5	37.1	40.3	58.3	60.6	45.3
% Conv.C_b^a→Py_b^a	0.0	7.8	74.3	88.8	100	100	100	0.0	11.1	12.6	23.1	28.4	40.9	50.3

Table 4.16 Pigment Content of Pea Puree Processed at 280°F and Stored for 18 Months at 68°F

Pigment	Pigment Content (μg/g)													
	Normal pH							Elevated pH						
	Unprocessed	Storage Time (Months) 0	2	3	6	12	18	Unprocessed	Storage Time (Months) 0	2	3	6	12	18
C_a	60.8	55.6	1.8	0.0	0.0	0.0	0.0	54.6	53.6	45.7	49.0	30.3	17.8	26.8
C_b	25.2	22.7	8.4	4.3	1.6	4.5	0.0	22.6	22.8	20.5	15.9	16.3	21.8	19.0
Py_a	3.6	11.0	68.1	65.5	62.4	65.1	62.6	3.2	6.0	13.7	9.1	25.1	32.8	18.7
Py_b	0.0	2.4	15.8	11.2	24.6	22.5	25.1	0.0	0.7	3.4	8.7	6.1	5.1	14.2
Total C	86.0	78.3	10.2	4.3	1.6	4.5	0.0	77.2	76.4	66.2	64.9	46.6	39.6	45.8
Total Py	3.6	13.4	83.9	87.6	87.0	87.6	87.7	3.2	6.7	17.1	17.8	31.2	37.9	32.9
Total C + Py	89.6	91.7	94.1	91.9	88.6	92.1	87.7	80.4	83.1	83.3	82.7	77.8	77.5	78.7
% Conv. C→Py	4.0	14.6	89.2	95.3	98.2	95.1	100	4.0	8.1	20.5	21.5	40.1	48.9	41.8
% Conv. $C_a \rightarrow Py_a$	5.6	16.5	97.4	100	100	100	100	5.5	10.1	23.1	15.7	45.3	64.8	41.1
% Conv. $C_b \rightarrow Py_b$	0.0	9.6	65.3	61.1	93.9	83.3	100	0.0	3.0	14.2	35.4	27.2	19.0	42.8

Table 4.17 Pigment Content of Pea Puree Processed at 300°F and Stored for 18 Months at 68°F

Pigment	Pigment Content (μg/g)													
	Normal pH							Elevated pH						
	Unprocessed	Storage Time (Months) 0	2	3	6	12	18	Unprocessed	Storage Time (Months) 0	2	3	6	12	18
C_a	60.8	51.9	4.0	1.2	3.0	0.0	0.0	54.6	51.8	46.2	42.1	35.2	22.5	27.2
C_b	25.2	24.1	8.0	7.7	5.5	0.0	0.0	22.6	21.2	19.0	16.8	18.7	22.5	22.4
Py_a	3.6	12.4	50.4	63.3	61.9	63.4	67.0	3.2	7.0	12.4	13.1	24.2	30.0	22.1
Py_b	0.0	2.4	17.5	17.5	21.8	25.4	24.4	0.0	1.7	3.2	6.3	5.0	7.0	10.5
Total C	86.0	76.0	12.0	8.9	8.5	0.0	0.0	77.2	73.0	65.2	58.9	53.9	45.0	49.6
Total Py	3.6	14.8	79.9	80.8	83.4	88.8	88.6	3.2	8.7	16.6	19.4	29.2	37.0	32.6
Total C + Py	89.6	90.8	91.9	89.7	91.9	88.8	88.6	80.4	81.7	81.8	78.3	83.1	82.0	82.2
% Conv. C→Py	4.0	16.3	86.9	90.1	90.8	100	100	4.0	10.7	20.3	24.8	35.1	45.1	39.7
% Conv. $C_a \rightarrow Py_a$	5.6	19.3	92.6	98.1	95.4	100	100	5.5	11.9	21.2	23.7	40.7	57.1	44.8
% Conv. $C_b \rightarrow Py_b$	0.0	9.1	68.6	69.4	79.6	100	100	0.0	7.4	14.4	27.2	21.1	23.7	31.9

Table 4.18 Pigment Content of Pea Puree Processed at 240°F and Stored for 18 Months at 37°F

Pigment	Pigment Content (μg/g)											
	Normal pH						Elevated pH					
	Unprocessed	Storage Time (Months) 0	3	6	12	18	Unprocessed	Storage Time (Months) 0	3	6	12	18
C_a	60.8	5.5	0.4	0.0	0.0	0.0	54.6	35.0	30.1	29.5	24.1	25.4
C_b	25.2	10.0	8.3	3.4	0.0	0.0	22.6	16.4	20.6	14.7	17.1	19.8
Py_a	3.6	61.1	63.8	62.8	63.0	65.9	3.2	21.9	26.1	27.4	31.0	28.1
Py_b	0.0	15.5	15.3	22.4	26.1	26.3	0.0	5.6	2.1	10.1	7.7	3.7
Total C	86.0	15.5	8.7	3.4	0.0	0.0	77.2	51.4	50.7	44.2	41.2	45.2
Total Py	3.6	76.6	79.1	85.2	89.1	92.2	3.2	27.5	28.2	37.5	38.7	31.8
Total C + Py	89.6	92.1	87.8	88.6	89.1	92.2	80.4	78.9	78.9	81.7	79.9	77.0
% Conv. $C \rightarrow Py$	4.0	83.2	90.1	96.2	100	100	4.0	34.9	35.7	45.9	48.4	41.3
% Conv. $C_a \rightarrow Py_a$	5.6	91.7	99.1	100	100	100	5.5	39.5	46.4	51.8	56.3	52.5
% Conv. $C_b \rightarrow Py_b$	0.0	60.8	64.8	86.8	100	100	0.0	25.5	9.3	40.7	31.0	15.7

Table 4.19 Pigment Content of Pea Puree Processed at 260°F and Stored for 18 Months at 37°F

Pigment	Pigment Content (μg/g)											
	Normal pH						Elevated pH					
	Unprocessed	Storage Time (Months) 0	3	6	12	18	Unprocessed	Storage Time (Months) 0	3	6	12	18
C_a	60.8	38.3	19.7	3.8	0.9	0.0	54.6	48.0	45.9	47.4	42.3	37.8
C_b	25.2	23.5	5.0	8.7	7.8	1.7	22.6	19.3	22.2	21.1	21.6	19.0
Py_a	3.6	24.7	46.3	59.0	64.8	64.4	3.2	10.2	12.8	10.3	12.4	17.3
Py_b	0.0	2.0	21.5	15.6	16.5	23.0	0.0	2.4	2.4	2.2	1.7	4.4
Total C	86.0	61.8	24.7	12.5	8.7	1.7	77.2	67.3	68.1	68.5	63.9	56.8
Total Py	3.6	26.7	67.8	74.6	81.3	87.4	3.2	12.6	15.2	12.5	14.1	21.7
Total C + Py	89.6	88.7	92.5	87.1	90.0	89.1	80.4	79.9	83.3	81.0	78.0	78.5
% Conv. $C \rightarrow Py$	4.0	30.1	73.3	85.6	90.3	98.1	4.0	15.8	18.3	15.4	18.1	27.7
% Conv. $C_a \rightarrow Py_a$	5.6	39.2	70.2	93.9	98.6	100	5.5	17.5	21.8	17.9	22.7	31.4
% Conv. $C_b \rightarrow Py_b$	0.0	7.8	81.1	64.2	67.9	93.1	0.0	11.1	9.8	9.4	7.3	18.8

Table 4.20 Pigment Content of Pea Puree Processed at 280°F and Stored for 18 Months at 37°F

Pigment	Pigment Content (μg/g)											
	Normal pH						Elevated pH					
	Unprocessed	Storage Time (Months) 0	3	6	12	18	Unprocessed	Storage Time (Months) 0	3	6	12	18
C_a	60.8	55.6	27.2	10.8	7.4	1.0	54.6	53.6	52.3	50.2	46.8	41.9
C_b	25.2	22.7	14.3	14.6	5.9	6.3	22.6	22.8	18.9	23.3	22.4	24.3
Py_a	3.6	11.0	43.6	53.6	55.4	61.6	3.2	6.0	6.0	6.8	10.9	13.0
Py_b	0.0	2.4	11.0	11.0	19.7	19.8	0.0	0.7	4.5	1.1	2.0	1.6
Total C	86.0	78.3	41.5	25.4	13.3	7.3	77.2	76.4	71.2	73.5	69.2	66.2
Total Py	3.6	13.4	49.9	64.6	75.1	81.4	3.2	6.7	10.5	8.9	12.9	14.6
Total C + Py	89.6	91.7	91.4	90.0	88.4	88.7	80.4	83.1	81.7	82.4	82.1	80.8
% Conv.C→Py	4.0	14.6	54.6	71.8	84.9	91.8	4.0	8.1	12.8	10.8	15.7	18.1
% Conv.C_a→Py_a	5.6	16.5	61.6	83.2	88.2	98.4	5.5	10.1	10.3	11.9	18.9	23.7
% Conv.C_b→Py_b	0.0	9.6	43.5	43.0	76.9	75.9	0.0	3.0	19.2	4.5	8.2	6.2

Table 4.21 Pigment Content of Pea Puree Processed at 300°F and Stored for 18 Months at 37°F

Pigment	Pigment Content (μg/g)											
	Normal pH						Elevated pH					
	Unprocessed	Storage Time (Months) 0	3	6	12	18	Unprocessed	Storage Time (Months) 0	3	6	12	18
C_a	60.8	51.9	25.2	14.5	6.9	1.9	54.6	51.8	52.5	47.8	51.4	46.4
C_b	25.2	24.1	12.5	13.3	4.9	4.0	22.6	21.2	21.5	19.3	21.8	21.9
Py_a	3.6	12.4	38.3	51.3	57.2	62.7	3.2	7.0	7.0	7.5	7.1	8.6
Py_b	0.0	2.4	12.1	14.3	22.7	21.1	0.0	1.7	1.5	3.7	1.2	2.7
Total C	86.0	76.0	37.7	27.8	11.8	5.9	77.2	73.0	74.0	67.1	73.2	68.3
Total Py	3.6	14.8	50.4	65.6	79.9	83.8	3.2	8.7	8.5	11.2	8.3	11.3
Total C + Py	89.6	90.8	88.1	93.4	91.7	89.7	80.4	81.7	82.5	78.3	81.5	79.6
% Conv.C→Py	4.0	16.3	57.2	70.2	87.1	93.4	4.0	10.7	10.3	14.3	10.2	14.2
% Conv.C→Py_a	5.6	19.3	60.3	78.0	89.2	97.1	5.5	11.9	11.8	13.6	12.1	15.6
% Conv.C_b→Py_b	0.0	9.1	49.2	51.8	82.2	84.1	0.0	7.4	6.5	16.1	5.2	11.0

Table 4.22 Pigment Content of Pea Puree Processed at 240°F and Stored for 18 Months at -10°F

Pigment	Pigment Content (μg/g)									
	Normal pH					Elevated pH				
	Unprocessed	Storage Time (Months) 0	6	12	18	Unprocessed	Storage Time (Months) 0	6	12	18
C_a	60.8	5.5	4.0	2.2	4.0	54.6	35.0	27.7	31.7	27.1
C_b	25.2	10.0	6.6	10.0	9.5	22.6	16.4	17.9	20.2	18.4
Py_a	3.6	61.1	64.3	62.1	61.6	3.2	21.9	28.7	26.8	31.9
Py_b	0.0	15.5	19.3	17.0	16.5	0.0	5.6	4.8	3.5	4.7
Total C	86.0	15.5	10.6	12.2	13.5	77.2	51.4	45.6	50.9	45.5
Total Py	3.6	76.6	83.6	79.1	78.2	3.2	27.5	33.5	31.3	36.6
Total C + Py	89.6	92.1	94.2	91.3	91.7	80.4	78.9	79.1	82.2	82.1
% Conv.C→Py	4.0	83.2	88.7	86.6	85.3	4.0	34.9	42.3	38.1	44.6
% Conv.C→Py_a	5.6	91.7	94.1	96.6	93.9	5.5	39.5	50.9	45.8	54.1
% Conv.C_b^a→Py_b	0.0	60.8	74.5	63.0	63.6	0.0	25.5	21.1	15.2	20.3

Table 4.23 Pigment Content of Pea Puree Processed at 260°F and Stored for 18 Months at -10°F

Pigment	Pigment Content (μg/g)									
	Normal pH					Elevated pH				
	Unprocessed	Storage Time (Months) 0	6	12	18	Unprocessed	Storage Time (Months) 0	6	12	18
C_a	60.8	38.3	45.1	43.0	39.1	54.6	48.0	41.4	51.3	46.4
C_b	25.2	23.5	20.3	17.8	21.8	22.6	19.3	20.1	21.8	21.1
Py_a	3.6	24.7	20.1	21.1	25.1	3.2	10.2	11.0	7.0	9.2
Py_b	0.0	2.0	5.2	6.8	4.6	0.0	2.4	2.1	1.5	2.1
Total C	86.0	61.8	65.4	60.8	60.9	77.2	67.3	66.5	73.1	67.5
Total Py	3.6	26.7	26.3	27.9	29.7	3.2	12.6	13.1	8.5	11.3
Total C + Py	89.6	88.7	91.7	88.7	90.6	80.4	79.9	79.6	81.6	78.8
% Conv.C→Py	4.0	30.1	28.7	31.4	32.8	4.0	15.8	16.5	10.4	14.3
% Conv.C→Py_a	5.6	39.2	30.8	32.9	39.1	5.5	17.5	20.9	12.0	16.5
% Conv.C_b^a→Py_b	0.0	7.8	20.4	27.6	17.4	0.0	11.1	9.5	6.4	9.1

Table 4.24 Pigment Content of Pea Puree Processed at 280°F and Stored for 18 Months at -10°F

Pigment	Pigment Content (μg/g)									
	Normal pH					Elevated pH				
	Unprocessed	Storage Time (Months) 0	6	12	18	Unprocessed	Storage Time (Months) 0	6	12	18
C_a	60.8	55.6	51.5	50.7	52.2	54.6	53.6	52.5	51.1	47.2
C_b	25.2	22.7	21.7	23.5	20.8	22.6	22.8	23.1	23.3	22.0
Py_a	3.6	11.0	12.3	11.6	11.6	3.2	6.0	5.7	7.3	9.2
Py_b	0.0	2.4	4.1	2.4	3.2	0.0	0.7	1.5	1.3	0.9
Total C	86.0	78.3	73.2	74.2	73.0	77.2	76.4	75.6	74.4	69.2
Total Py	3.6	13.4	16.4	14.0	14.8	3.2	6.7	7.2	8.6	10.1
Total C + Py	89.6	91.7	89.6	88.2	87.8	80.4	83.1	82.8	83.0	79.3
% Conv. $C \rightarrow Py$	4.0	14.6	18.3	15.9	16.8	4.0	8.1	8.7	10.4	12.7
% Conv. $C \rightarrow Py_a$	5.6	16.5	19.3	17.5	18.2	5.5	10.1	9.8	12.5	16.3
% Conv. $C_b^a \rightarrow Py_b$	0.0	9.6	15.9	9.3	13.3	0.0	3.0	6.1	5.3	3.9

Table 4.25 Pigment Content of Pea Puree Processed at 300°F and Stored for 18 Months at -10°F

Pigment	Pigment Content (μg/g)									
	Normal pH					Elevated pH				
	Unprocessed	Storage Time (Months) 0	6	12	18	Unprocessed	Storage Time (Months) 0	6	12	18
C_a	60.8	51.9	52.3	53.0	53.3	54.6	51.8 4	47.3	50.4	50.0
C_b	25.2	24.1	24.1	23.1	23.5	22.6	21.2	22.6	23.2	20.3
Py_a	3.6	14.2	14.0	11.4	13.3	3.2	7.0	8.2	5.6	7.7
Py_b	0.0	2.4	1.9	2.6	3.3	0.0	1.7	0.3	0.0	3.0
Total C	86.0	76.0	76.4	78.1	76.8	77.2	73.0	69.9	73.6	70.3
Total Py	3.6	14.8	15.9	14.0	16.6	3.2	8.7	8.5	5.6	10.7
Total C + Py	89.6	90.8	92.3	92.1	93.4	80.4	81.7	78.4	79.2	81.0
% Conv. $C \rightarrow Py$	4.0	16.3	17.2	15.2	17.8	4.0	10.7	10.9	7.1	13.2
% Conv. $C \rightarrow Py_a$	5.6	19.3	21.1	17.7	20.0	5.5	11.9	14.8	10.0	13.3
% Conv. $C_b^a \rightarrow Py_b$	0.0	9.1	7.3	10.1	12.3	0.0	7.4	1.3	0.0	12.9

11. Colour and Colour-Difference Measurements

11.1 Standardisation of Hunterlab Colour and Colour-Difference Meter

The instrument used for objective colour measurements, a Hunterlab Model D25 Colour and Colour-Difference Meter (Hunter Associates Laboratory, McLean, Virginia, U.S.A.), was supplied with five 4" x 4" ceramic tiles used for standardisation. The tiles were calibrated in terms of C.I.E. tristimulus values X, Y and Z by the C.S.I.R.O. National Standards Laboratory, Sydney (Edwards 1963), by measuring the diffuse spectral reflectance of each tile relative to that of freshly prepared magnesium oxide. Hunterlab L, a and b values of the tiles computed from the C.I.E. tristimulus values, together with the L, a and b values assigned by the Hunter Laboratories, are given in Table 4.26 (from Edwards 1963).

Table 4.26 L, a and b Values of Reference Tiles

Tile	Code	L Value	a Value	b Value
White	P.T.13337	94.2 (95.1)*	-1.7(-0.9)	2.3(2.2)
Grey	P.T.13338	21.9(22.4)	-0.1(-0.7)	-1.7(-1.5)
Pink	P.T.13340	67.9(67.9)	16.3(16.2)	10.2(8.6)
Green	P.T.13341	59.7(60.7)	-16.4(-16.5)	7.4(6.9)
Cream	P.T.13342	83.0(84.0)	-5.0(-5.6)	26.7(26.7)

* Bracketed figures refer to values assigned by Hunter Laboratory.

11.2 Effect of Cell Depth on L, a and b Values

The Hunterlab instrument was standardised using the green tile (values L = 59.7, a = -16.4, b = 7.4) according to instructions in the instrument manual. Cylindrical plastic cells, 2 inch diameter of varying depth, were filled with pea puree prepared by blending commercial canned peas with the canning brine in an Atomix blender. The cells containing

puree were covered with a thin plate of optical glass taking care to avoid inclusion of air bubbles, and duplicate L, a and b values for each cell measured according to the instruction manual. Average values are presented in Table 4.27.

Table 4.27 L, a and b Values of Pureed Canned Peas in Cells of Varying Depth

Cell Depth (inch)	L Value	a Value	b Value
1/4	46.5	-4.0	24.6
1/2	46.4	-4.0	24.6
3/4	46.4	-4.0	24.6
1	46.4	-4.0	24.6
1½	46.4	-4.0	24.6

12. Colour Measurements on Pea Puree

12.1 Processed Puree

Samples of normal and elevated pH pea puree prepared before and after H.T.S.T. processing were analysed by the Hunterlab instrument. Duplicate measurements were taken in ½ inch deep plastic cells, after the instrument was calibrated using the green tile. Munsell values were determined by matching puree samples with removable colour chips from the Munsell Book of Colour (1965) under standard illumination according to the instruction booklet.

Visual ranking of the pea puree in order of "desirable green pea colour" was performed by a panel of four judges on coded puree samples in small glass beakers under artificial illumination. The colour matching unit, using fluorescent and tungsten lamps, produced illumination similar to North Sky daylight of approximately 6500°K black body radiation

having C.I.E. chromaticity co-ordinates x = 0.314, y = 0.324 and z = 0.362. Results are presented in Table 4.28.

Table 4.28 L, a and b Values and Visual Rank Score on Pea Purees of Different pH Processed by H.T.S.T. Methods

Process Temperature (°F)	Initial Puree pH	Hunterlab Values			Visual Rank *					
		L	a	b	A	B	C	D	Mean	Order
Unprocessed	6.95	45.1	-20.0	25.5	1	1	1	1	1	1
240	"	41.0	- 5.7	22.5	10	10	10	10	10	10
260	"	39.5	-13.5	21.9	8	9	8	8	8.25	8
280	"	40.8	-16.5	23.0	3	3	3	2	2.75	3
300	"	41.7	-16.6	23.3	2	2	2	3	2.25	2
Unprocessed	8.45	52.8	-19.4	26.2	4	4	4	4	4	4
240	"	46.6	-11.7	22.3	9	8	9	9	8.75	9
260	"	50.8	-17.3	24.9	7	7	7	7	7	7
280	"	52.1	-18.6	25.9	5	6	5	5	5.25	5
300	"	51.9	-18.5	25.7	6	5	6	6	5.75	6

* Figure 1 indicates most desirable greenness
Figure 10 least desirable greenness.

Correlation coefficients were calculated relating visual rank score with the Hunterlab indices $^{-a}/_{b}$, $^{-a}/_{L}$ and $(a^2+b^2)^{\frac{1}{2}}$, and with pigment determinations for each sample. The values are given in Tables 3.10 and 3.11 (Discussion).

12.2 Stored Puree

Hunterlab L, a and b values for processed pea puree stored for 18 months at 68°F, 37°F and -10°F are presented in Tables 4.29 to 4.40. The derived colour indices $^{-a}/_{b}$, $^{-a}/_{L}$ and $(a^2+b^2)^{\frac{1}{2}}$ are also presented. Multiple correlation coefficients and regression equations relating percent conversion of chlorophylls to pheophytins with colour indices

$-a/b$, $-a/L$ and $(a^2+b^2)^{\frac{1}{2}}$ for normal and elevated pH puree after 18 months storage were calculated by computer (IBM CDC 3600, Michigan State University Program, CORE Routine) at the C.S.I.R.O. Division of Computing Research, Canberra. Results are presented in Table 3.12 (Discussion).

Table 4.29 Objective Colour Indices of Pea Puree Processed at 240°F and Stored for 18 Months at 68°F

Colour Index	Normal pH							Elevated pH						
	Unprocessed	Storage Time (Months) 0	2	3	6	12	18	Unprocessed	Storage Time (Months) 0	2	3	6	12	18
L	45.1	41.0	46.2	46.2	46.2	46.2	46.2	52.8	46.6	48.4	48.5	48.6	48.6	48.9
a	-20.0	-5.7	-4.3	-4.1	-4.0	-4.0	-4.0	-19.4	-11.7	-10.4	-10.0	- 9.0	-7.0	-6.1
b	25.5	22.5	22.5	24.0	24.0	24.0	24.0	26.2	22.3	22.5	22.6	22.6	22.6	23.6
$-a/b$	0.784	0.253	0.179	0.170	0.167	0.167	0.167	0.740	0.525	0.462	0.442	0.398	0.310	0.258
$-a/L$	0.443	0.139	0.093	0.088	0.087	0.087	0.087	0.366	0.251	0.215	0.206	0.185	0.144	0.125
$(a^2+b^2)^{\frac{1}{2}}$	32.40	23.21	24.38	24.35	24.33	24.33	24.33	32.61	25.18	24.79	24.72	24.33	23.66	24.37

Table 4.30 Objective Colour Indices of Pea Puree Processed at 260°F and Stored for 18 Months at 68°F

Colour Index	Normal pH							Elevated pH						
	Unprocessed	Storage Time (Months) 0	2	3	6	12	18	Unprocessed	Storage Time (Months) 0	2	3	6	12	18
L	45.1	39.5	46.3	46.5	46.6	46.5	46.6	52.8	50.8	51.9	51.7	51.7	51.4	51.0
a	-20.0	-13.5	-4.7	-4.4	-4.4	-4.4	-4.2	-19.4	-17.3	-15.2	-14.2	-12.3	-9.1	-7.5
b	25.5	21.9	24.0	24.1	24.0	24.0	24.8	26.2	24.9	24.4	24.2	24.1	23.7	24.4
$-a/b$	0.784	0.616	0.195	0.183	0.183	0.183	0.169	0.740	0.695	0.623	0.587	0.510	0.384	0.307
$-a/L$	0.443	0.342	0.102	0.095	0.094	0.095	0.090	0.496	0.341	0.293	0.275	0.238	0.177	0.147
$(a^2+b^2)^{\frac{1}{2}}$	32.40	25.73	24.45	24.49	24.40	24.62	25.15	32.61	30.32	28.75	27.52	27.06	25.39	25.52

Table 4.31 Objective Colour Indices of Pea Puree Processed at 280°F and Stored for 18 Months at 68°F

Colour Index	Normal pH							Elevated pH						
	Unprocessed	Storage Time (Months) 0	2	3	6	12	18	Unprocessed	Storage Time (Months) 0	2	3	6	12	18
L	45.1	40.8	46.4	46.5	46.6	46.8	46.8	52.8	52.1	52.7	52.5	52.8	52.9	52.8
a	-20.0	-16.5	-5.0	-4.7	-4.6	-4.5	-4.4	-19.4	-18.6	-16.0	-14.6	-12.7	-9.3	-8.0
b	25.5	23.0	23.8	24.0	24.1	24.2	24.9	26.2	25.9	24.7	24.3	24.5	24.4	25.1
$-a/b$	0.784	0.717	0.210	0.196	0.191	0.186	0.179	0.740	0.718	0.648	0.601	0.518	0.381	0.319
$-a/L$	0.443	0.404	0.108	0.101	0.099	0.096	0.094	0.366	0.357	0.304	0.278	0.241	0.176	0.152
$(a^2+b^2)^{\frac{1}{2}}$	32.40	28.30	24.32	24.45	24.45	24.62	25.29	32.61	31.89	29.43	28.35	27.60	26.12	26.34

Table 4.32 Objective Colour Indices of Pea Puree Processed at 300°F and Stored for 18 Months at 68°F

Colour Index	Normal pH							Elevated pH						
	Unprocessed	Storage Time (Months) 0	2	3	6	12	18	Unprocessed	Storage Time (Months) 0	2	3	6	12	18
L	45.1	41.7	46.6	46.9	46.8	47.3	47.0	52.8	51.9	52.8	52.9	52.9	53.2	53.2
a	-20.0	-16.6	-5.0	-4.7	-4.6	-4.6	-4.5	-19.4	-18.5	-16.2	-15.1	-13.0	-9.5	-8.2
b	25.5	23.3	24.1	24.2	24.2	24.4	25.0	26.2	25.7	24.7	24.6	24.6	24.6	25.3
$-a/b$	0.784	0.712	0.207	0.194	0.190	0.189	0.180	0.740	0.720	0.656	0.614	0.528	0.386	0.324
$-a/L$	0.443	0.398	0.107	0.100	0.098	0.097	0.096	0.366	0.356	0.307	0.285	0.246	0.179	0.154
$(a^2+b^2)^{\frac{1}{2}}$	32.40	28.61	24.62	24.65	24.64	24.83	25.41	32.61	31.65	29.54	28.86	27.82	26.37	26.60

Table 4.33 Objective Colour Indices of Pea Puree Processed at 240°F and Stored for 18 Months at 37°F

Colour Index	Normal pH						Elevated pH					
	Unprocessed	Storage Time (Months) 0	3	6	12	18	Unprocessed	Storage Time (Months) 0	3	6	12	18
L	45.1	41.0	46.2	46.1	46.2	46.4	52.8	46.6	48.5	48.8	48.0	47.9
a	-20.0	-5.7	-5.0	-4.7	-4.3	-4.1	-19.4	-11.7	-11.8	-11.5	-11.1	-11.0
b	25.5	22.5	24.2	24.0	24.0	24.0	26.2	22.3	22.6	22.8	22.3	22.8
$-a/b$	0.784	0.253	0.207	0.196	0.179	0.170	0.740	0.525	0.522	0.504	0.498	0.482
$-a/L$	0.443	0.139	0.108	0.102	0.093	0.088	0.366	0.251	0.243	0.236	0.231	0.230
$(a^2+b^2)^{\frac{1}{2}}$	32.40	23.21	24.71	22.41	24.38	24.35	32.61	25.18	25.50	25.53	24.91	25.32

Table 4.34 Objective Colour Indices of Pea Puree Processed at 260°F and Stored for 18 Months at 37°F

Colour Index	Normal pH						Elevated pH					
	Unprocessed	Storage Time (Months) 0	3	6	12	18	Unprocessed	Storage Time (Months) 0	3	6	12	18
L	45.1	39.5	45.2	45.8	46.0	45.9	52.8	50.8	52.0	52.3	51.9	51.0
a	-20.0	-13.5	-8.5	-6.3	-5.2	-4.6	-19.4	-17.3	-17.1	-16.8	-16.5	-15.7
b	25.5	21.9	23.9	24.1	23.8	24.3	26.2	24.9	24.8	24.9	24.5	24.8
$-a/b$	0.784	0.616	0.357	0.261	0.218	0.189	0.740	0.695	0.690	0.675	0.673	0.633
$-a/L$	0.443	0.342	0.188	0.138	0.113	0.100	0.366	0.341	0.329	0.321	0.318	0.308
$(a^2+b^2)^{\frac{1}{2}}$	32.40	25.73	25.37	24.91	24.36	24.73	32.61	30.32	30.13	30.03	29.54	29.35

Table 4.35 Objective Colour Indices of Pea Puree Processed at 280°F and Stored for 18 Months at 37°F

Colour Index	Normal pH						Elevated pH					
	Unprocessed	Storage Time (Months) 0	3	6	12	18	Unprocessed	Storage Time (Months) 0	3	6	12	18
L	45.1	40.8	45.0	45.6	46.0	45.8	52.8	52.1	53.0	52.9	52.9	52.2
a	-20.0	-16.5	-10.7	-7.6	-5.8	-5.0	-19.4	-18.6	-18.1	-17.7	-17.4	-16.9
b	25.5	23.0	23.9	24.0	24.1	24.1	26.2	25.9	25.4	25.2	25.0	25.3
$-a/b$	0.784	0.717	0.448	0.317	0.241	0.207	0.740	0.718	0.713	0.702	0.696	0.668
$-a/L$	0.443	0.404	0.238	0.167	0.126	0.109	0.366	0.357	0.342	0.335	0.329	0.324
$(a^2+b^2)^{\frac{1}{2}}$	32.40	28.30	26.18	25.18	24.79	24.62	32.61	31.89	31.19	30.79	30.46	30.42

Table 4.36 Objective Colour Indices of Pea Puree Processed at 300°F and Stored for 18 Months at 37°F

Colour Index	Normal pH						Elevated pH					
	Unprocessed	Storage Time (Months) 0	3	6	12	18	Unprocessed	Storage Time (Months) 0	3	6	12	18
L	45.1	41.7	44.9	45.5	46.0	45.6	52.8	51.9	52.9	53.1	52.7	52.6
a	-20.0	-16.6	-10.3	-7.7	-5.7	-5.2	-19.4	-18.5	-18.2	-17.8	-17.5	-17.0
b	25.5	23.3	23.8	24.1	24.0	24.2	26.2	25.7	25.4	25.2	25.1	25.6
$-a/b$	0.784	0.712	0.433	0.320	0.238	0.215	0.740	0.720	0.717	0.706	0.697	0.664
$-a/L$	0.443	0.398	0.229	0.169	0.124	0.114	0.366	0.356	0.344	0.335	0.332	0.323
$(a^2+b^2)^{\frac{1}{2}}$	32.40	28.61	25.93	25.30	24.67	24.75	32.61	31.65	31.25	30.85	30.59	30.73

Table 4.37 Objective Colour Indices of Pea Puree Processed at 240°F and Stored for 18 Months at -10°F

Colour Index	Normal pH					Elevated pH				
	Unprocessed	Storage Time (Months) 0	6	12	18	Unprocessed	Storage Time (Months) 0	6	12	18
L	45.1	41.0	40.6	41.0	40.6	42.8	46.6	45.7	46.5	44.8
a	-20.0	-5.7	-5.7	- 5.7	-5.7	-19.4	-11.7	-11.5	-11.9	-11.5
b	25.5	22.5	22.5	22.5	22.6	26.2	22.3	22.3	22.5	22.3
$-a/b$	0.784	0.253	0.253	0.253	0.252	0.740	0.525	0.516	0.529	0.516
$-a/L$	0.443	0.139	0.140	0.139	0.140	0.366	0.251	0.252	0.256	0.257
$(a^2+b^2)^{\frac{1}{2}}$	32.40	23.21	23.21	23.21	23.24	32.61	25.18	25.09	25.46	25.09

Table 4.38 Objective Colour Indices of Pea Puree Processed at 260°F and Stored for 18 Months at -10°F

Colour Index	Normal pH					Elevated pH				
	Unprocessed	Storage Time (Months) 0	6	12	18	Unprocessed	Storage Time (Months) 0	6	12	18
L	45.1	39.5	40.1	40.3	39.8	52.8	50.8	49.8	49.9	49.8
a	-20.0	-13.5	-13.5	-13.9	-14.0	-19.4	-17.3	-17.0	-17.4	-17.3
b	25.5	21.9	22.3	22.3	23.2	26.2	24.9	24.8	24.7	25.4
$-a/b$	0.784	0.616	0.605	0.623	0.603	0.740	0.695	0.685	0.704	0.681
$-a/L$	0.443	0.342	0.337	0.345	0.352	0.366	0.341	0.498	0.349	0.347
$(a^2+b^2)^{\frac{1}{2}}$	32.40	25.73	26.07	26.28	27.09	32.61	30.32	30.07	30.21	30.73

Table 4.39 Objective Colour Indices of Pea Puree Processed at 280°F and Stored for 18 Months at -10°F

Colour Index	Normal pH					Elevated pH				
	Unprocessed	Storage Time (Months) 0	6	12	18	Unprocessed	Storage Time (Months) 0	6	12	18
L	45.1	40.8	40.8	41.2	40.2	52.8	52.1	51.9	50.9	51.8
a	-20.0	-16.5	-16.4	-16.7	-16.8	-19.4	-18.6	-18.3	-18.1	-18.0
b	25.5	23.0	23.0	23.2	23.6	26.2	28.9	25.7	25.2	26.2
$-a/b$	0.784	0.717	0.713	0.720	0.712	0.740	0.718	0.712	0.718	0.687
$-a/L$	0.443	0.404	0.402	0.405	0.418	0.366	0.357	0.353	0.356	0.347
$(a^2+b^2)^{\frac{1}{2}}$	32.40	28.30	28.25	28.58	28.97	32.61	31.89	31.55	31.03	31.78

Table 4.40 Objective Colour Indices of Pea Puree Processed at 300°F and Stored for 18 Months at -10°F

Colour Index	Normal pH					Elevated pH				
	Unprocessed	Storage Time (Months) 0	6	12	18	Unprocessed	Storage Time (Months) 0	6	12	18
L	45.1	41.7	41.8	41.9	41.4	52.8	51.9	52.3	52.6	52.0
a	-20.0	-16.6	-16.5	-16.8	-16.8	-19.4	-18.5	-18.4	-18.7	-18.4
b	25.5	23.3	23.5	23.6	24.0	26.2	25.7	25.8	25.9	26.4
$-a/b$	0.784	0.712	0.702	0.712	0.700	0.740	0.720	0.713	0.722	0.697
$-a/L$	0.443	0.398	0.395	0.401	0.406	0.366	0.356	0.352	0.356	0.354
$(a^2+b^2)^{\frac{1}{2}}$	32.40	28.61	28.72	28.97	29.29	32.61	31.65	32.25	31.96	32.18

13. pH of Pea Puree

The pH of puree samples after storage was measured with a Pye Dynacap pH meter on a slurry of 5-10g puree with 20-25ml boiled distilled water. The pH meter was standardised with buffer solutions of known pH (6.99 and 9.00 at 68°F). Results are presented in Table 4.41.

Table 4.41 pH Values of Processed and Stored Pea Puree

Storage Time	Storage Temp. (°F)	Normal pH				Elevated pH			
		Process Temp. (°F)				Process Temp. (°F)			
		240	260	280	300	240	260	280	300
Unprocessed	-	6.95	6.95	6.95	6.95	8.45	8.45	8.45	8.45
Processed	-	6.47	6.80	7.04	7.16	8.25	8.34	8.40	8.49
2 months	68	6.38	6.54	6.69	6.72	8.18	8.25	8.26	8.28
3 "	"	6.31	6.50	6.60	6.67	8.02	8.18	8.22	8.26
6 "	"	6.25	6.48	6.55	6.57	7.97	8.14	8.24	8.26
12 "	"	6.18	6.43	6.49	6.48	7.88	7.95	8.19	8.20
18 "	"	6.09	6.14	6.28	6.30	7.79	7.88	7.95	8.10
3 "	37	6.39	6.69	6.81	6.95	8.25	8.28	8.30	8.30
6 "	"	6.31	6.68	6.79	6.86	8.24	8.20	8.25	8.32
12 "	"	6.27	6.56	6.77	6.84	8.19	8.21	8.27	8.27
18 "	"	6.30	6.58	6.69	6.77	8.11	8.18	8.23	8.28
6 "	-10	6.52	6.61	6.92	7.05	8.21	8.33	8.35	8.36
12 "	"	6.46	6.61	6.92	7.05	8.21	8.33	8.35	8.36
18 "	"	6.39	6.52	6.70	6.95	8.16	8.22	8.30	8.34

PART I B. ISOLATION AND PURIFICATION OF PIGMENTS FROM H.T.S.T. PROCESSED PEA PUREE

14. Thin-Layer Chromatographic Method for the Separation of Chlorophyll Pigments

The following method of thin-layer chromatography, used in various stages for the isolation and purification of chlorophyll-type pigments, was a modification of the technique of Bacon (1965a,b).

(i) Adsorbent: Whatman Thin-Layer Chromedia CC41 Microgranular Cellulose powder.

(ii) Preparation of Layer: Glass plates for thin-layer chromatography, 20cm x 20cm, were degreased with chloroform, thoroughly washed with soap and hot water, rinsed in tap water and distilled water, and allowed to dry at room temperature. Before use, the plates were wiped with clean tissue paper. 50g cellulose powder was slurried with approximately 135ml distilled water in a 150ml beaker, the mixture stirred continuously for 5-10 minutes to remove air bubbles, the slurry poured into the barrel of a "Desaga" thin-layer spreader and the adsorbent spread over 5 plates at a thickness of approximately 0.5mm.

(iii) Drying of Plates: Plates were allowed to partially dry in a stream of air at room temperature until the adsorbent surface lost its gloss, placed horizontally in an oven at 105°C for 25 minutes, then vertically for 20 minutes, removed and allowed to cool over dry silica gel in a desiccator.

(iv) Application of Pigment: Pigment solutions were applied to the prepared plates in a darkened room using the apparatus described by Monteiro (1965). This consisted of a small glass reservoir 35mm x 13mm tapering

at one end to an opening 2.5mm diameter. To the opposite end, at right angles to the long axis of the reservoir, was attached a glass rod handle 70mm x 3.5mm. A hole 4mm diameter in the reservoir wall about 19mm from the narrow end allowed the introduction of pigment solution. The solution was applied using glass capillary tube 70mm x 0.5mm diameter bent to an angle of 40° about 30mm from one end. The longer part of the capillary (40mm), dipped into the solution in the reservoir, while the shorter portion, positioned so that it was perpendicular to the surface of a horizontal plate, rested against the adsorbent surface. The plate was moved along a flat surface so that the capillary tube streaked the pigment solution along a line about 2cm from one edge of the plate. When sufficient pigment had been applied, the adsorbent on the 2 sides of the plate at right angles to the pigment streak was scraped away using a razor blade, leaving a straight adsorbent edge about 2mm from the side of the glass plate. This prevented side effects during development of the chromatogram.

(v) Development: Plates were developed in closed glass tanks lined with filter paper containing about 100ml solvent. For normal development, use was made of Bacon's solvent A containing petroleum ether (b.p.60-80°C): acetone: n-propanol in the ratio 90:10:0.45v/v/v. Solvent B, containing 20%v/v acetone in petroleum ether (b.p.60-80°C) was used for resolution of slower moving components. Before development, the tanks were allowed to equilibrate with the solvent for at least 30 minutes. During chromatogram development, the tanks were covered with black polythene to avoid photodecomposition of the pigments. When the solvent had travelled 15cm, the plates were removed from the tank and allowed to dry for 10 minutes at room temperature in the dark.

(vi) Elution: Pigment bands were detected in daylight or as fluorescent streaks under a UV lamp with "Woods" glass filter, and marked with a metal scriber. Marked areas were scraped off with a nickel spatula into a small sintered glass funnel attached by rubber stopper to an evacuated Quickfit test tube. Pigment was extracted from the adsorbent with acetone or diethyl ether.

(vii) Spectrophotometry: Visible spectra of separated pigments in acetone or ether, using matched 1cm silica cuvettes (capacity c.a.3ml) or matched 4cm "Suprasil" silica micro cells (capacity c.a.1.8ml), were recorded on a Unicam S.P.800 spectrophotometer and absorbances measured in a Unicam S.P.600 spectrophotometer.

15. Pigments from Elevated pH Pea Puree

15.1 Storage Temperature 68°F

About 40g puree (process temperature 300°F) was extracted repeatedly with cold acetone in an Atomix blender, the pulp filtered and washed with 80% acetone, diethyl ether was added, then 10% sodium chloride added slowly with shaking to phase the pigments into the ether layer. When the phases had separated, the aqueous acetone layer was run off, extracted with ether, and the combined ether extracts scrubbed through sodium chloride solution and distilled water to remove residual acetone. The ether solution was dried over anhydrous sodium sulphate in preparation for thin-layer chromatography on cellulose. The visible spectrum of the gross extract in diethyl ether is presented in Graph 12.

The diethyl ether extract was evaporated to a small volume in a rotary vacuum evaporator, and aliquots of approximately 0.5-2.0ml streaked

along the baseline of a series of 0.5mm cellulose thin-layer plates. The plates were placed into development tanks lined with filter paper and equilibrated for 30 minutes with Bacon's solvent A prior to chromatography. The plates were developed until the solvent front had moved 15cm from the pigment streak (about 12.5-14 minutes), then removed from the tanks and allowed to dry for 10 minutes at room temperature in the dark.

The plates were examined under daylight and UV light, the outlines of the bands marked by scratching the adsorbent with a metal pin, and the average distances travelled by each band recorded. The results are presented in Table 4.42.

Table 4.42 Bands Separated by T.L.C. on Cellulose from Elevated pH Pea Puree Stored 18 Months at 68°F

Band	Colour	Colour under UV	Distance Travelled (cm)
E 1	yellow	bluish-white	14.5[1] - 15.0[2]
E 2	grey	dark red	12.5 - 13.5
E 3	yellow	lemon green	11.5 - 12.5
E 4	yellow-green	red	11.0 - 11.5
E 5	greenish-grey	red	10.0 - 11.0
E 6	blue-green	pink	9.25- 9.75
E 7	yellow-green	red	7.5 - 8.5
E 8	green	pink	6.25- 6.75
E 9	pale yellow	pale green	5.0 - 5.5
E 10	green	orange-pink	4.0 - 4.75
E 11	faint green	faint pink	2.5 - 3.5
E 12	barely visible	faint pink	1.25- 1.75

1 = Average distance (cm) travelled by trailing edge of band

2 = Average distance (cm) travelled by leading edge of band

Each band was scraped as cleanly as possible from several plates, and the pooled adsorbent corresponding to each band extracted with acetone in

a sintered funnel. The pooled extracts were stored at -20°F until further purified by column and/or thin-layer chromatography.

Fraction E1 was evaporated to dryness in a stream of nitrogen, dissolved in a minimum of petroleum ether (b.p.40-60°C) and adsorbed on to a column of aluminium oxide, 15cm x 1.5cm (chromatographic grade) prepared by pouring dry alumina in a fine stream into a column of petroleum ether and gently tapping the column to aid settling and exclude air bubbles (Edwards 1963). The top of the column was covered with a disc of filter paper, solvent was run through the column, and the pigment solution was chromatographed with petroleum ether followed by 1% acetone in petroleum ether as the developing solvents. The major yellow band was dug out of the column, the pigment dissolved in petroleum ether (b.p.40-60°C) and n-hexane (spectroscopic grade) and spectra recorded (Graph 16).

Fraction E2 was dissolved in petroleum ether (b.p.60-80°C) and chromatographed on a wet-packed column of sugar/cellulose (30cm x 1.5cm). The column was developed with petroleum ether (b.p.60-80°C) and then 0.5% n-propanol in petroleum ether. The major grey band (brick-red fluorescence) was dug out, the pigment eluted with diethyl ether and the spectrum recorded (Graph 17).

Fraction E3 was dissolved in petroleum ether (b.p.60-80°C), and chromatographed on a wet-packed column of sugar/cellulose (35cm x 2.5cm) with petroleum ether as development solvent. The major yellow band (green fluorescence) was dug out, eluted with acetone, transferred to petroleum ether (b.p.40-60°C) and chromatographed on a wet-packed column of aluminium oxide (30cm x 1.5cm). The column was washed with petroleum

ether, 5% acetone, and 10% acetone in petroleum ether. At this point it was noticed that a colourless, strongly fluorescent band (blue under UV) travelled with the solvent front ahead of the main coloured band, which was still fixed near the top of the column. The colourless band was not collected. Upon development with 15% acetone in petroleum ether, three bands were formed in order of increasing absorption, E3.1 (pale yellow), E3.2 (pale lemon yellow) and the major, least sorbed E3.3 (golden yellow). The bands were dug out, the pigments eluted with acetone and transferred to petroleum ether (b.p.40-60°C) or n-hexane, and their spectra recorded (Graph 16).

Fraction E4 was dissolved in petroleum ether (b.p.60-80°C) and chromatographed on a wet-packed column of sugar/cellulose (30cm x 2.5cm). Upon development with petroleum ether and 0.5% n-propanol in petroleum ether, two bands were formed, E4.1 (diffuse yellow-green) and the major band E4.2 (yellow-green). Band E4.1 was eluted with acetone, transferred to petroleum ether (b.p.40-60°C), and chromatographed on a wet-packed column of aluminium oxide (25cm x 1.5cm) with petroleum ether, and then 5% acetone in petroleum ether as developing solvents. It was noticed that two colourless, fluorescent bands travelled close to the solvent front, while the main coloured band remained near the top of the column. The first band was narrow and fluoresced blue under UV light, while the second was more diffuse and gave a paler blue fluorescence. Neither band was collected. The column was developed with 10%, 15% and finally 20% acetone in petroleum ether, and two yellow bands were formed, E4.11 and E4.12. The pigments were eluted with acetone, transferred to petroleum ether (b.p.40-60°C) and their spectra recorded (Graph 19).

Band E4.2 was eluted with acetone, transferred to petroleum ether (b.p.60-80°C) and chromatographed on a wet-packed column of sugar/cellulose (25cm x 1.5cm) with 0.5% n-propanol in petroleum ether as developing solvent. The yellow-green band was eluted with diethyl ether and the spectrum recorded (Graph 18).

Fraction E5 was dissolved in petroleum ether (b.p.60-80°C) and chromatographed on a wet-packed column of sugar/cellulose (35cm x 2.5cm) developed with 1% n-propanol in petroleum ether. The major greenish-grey zone (dark red fluorescence) was eluted with diethyl ether, and rechromatographed on thin layers of cellulose with solvent A. The major band was scraped off, the pigment eluted with diethyl ether and the spectrum recorded (Graph 17).

Fraction E6 was dissolved in diethyl ether and chromatographed on thin layers of cellulose with solvent A. The blue-green band (red fluorescence) which appeared homogeneous was scraped off and the pigment eluted with acetone. Spectra were recorded in acetone and diethyl ether (Graph 20).

Fraction E7 was dissolved in petroleum ether (b.p.60-80°C) and chromatographed on a wet-packed column of sugar/cellulose (20cm x 1.5cm). Upon development with 0.5% and then 1% n-propanol in petroleum ether, 2 bands separated, the major band E7.1 (yellow-green) and a minor yellow component E7.2. The bands were eluted with acetone and their spectra recorded (Graphs 18, 19).

Fractions E8,E9,E10,E11 and E12 were dissolved in diethyl ether and chromatographed separately on thin layers of cellulose with solvent A or solvent B. The pigments were eluted with acetone or diethyl ether and their spectra recorded (Graphs 18,19,20,21).

The absorption maxima of separated pigment fractions in various solvents are presented in Table 4.43.

Table 4.43 Pigments from Elevated pH Pea Puree Stored for 18 Months at 68°F

Band	Colour	Absorption Maxima (nm)	Solvent	Identity
E1	yellow	424,450,478	n-hexane	β-carotene
		424,448,476	P.E.(bp40-60°C)	
E2	grey	409,470,506,534,562,609,666	diethyl ether	pheophytin a
E3.1	pale yellow	398,422,439,468	P.E.(bp40-60°C)	unidentified
E3.2	lemon yellow	418,439,468	n-hexane	"
E3.3	yellow	(396),422,446,476	n-hexane	lutein
		(396),420,445,474	P.E.(bp40-60°C)	
E4.11	yellow	400,421,447	P.E.(bp40-60°C)	unidentified
E4.12	yellow	403,424,451	P.E.(bp40-60°C)	"
E4.2	yellow-green	412,434,524,556,600,653	diethyl ether	pheophytin b
E5	grey	401,502,531,560,609,664	diethyl ether	unidentified
		400,501,529,558,607,662	acetone	(Pigment X)
E6	blue-green	410,429,500,533,577,615,660	diethyl ether	chlorophyll a
E7.1	greenish-yellow	427,524,561,599,653	diethyl ether	unidentified
		426,523,559,596,651	acetone	(Pigment Y)
E7.2	yellow	423,449,474	acetone	unidentified
E8	green	430,454,548,569,594,642	diethyl ether	chlorophyll b
E9	pale yellow	400,423,450	acetone	unidentified
E10	green	420,444,536,582,630	diethyl ether	unidentified
		422,446,582,631	acetone	(Pigment Z)
E11	green	422,446,582,630	acetone	unidentified
E12	barely visible	400,500,530,560,617,661	acetone	"

15.2 Storage Temperature -10°F

Pigment was extracted with acetone from puree processed at 300°F and stored 18 months, transferred to diethyl ether and chromatographed on thin layers of cellulose with solvent A. Separated bands were pooled and

pigments purified by further chromatography (column or TLC) as outlined previously. Pigment identification was based on absorption spectra and chromatographic behaviour with authentic samples. Results are presented in Table 4.44. The gross pigment spectrum is given in Graph 12.

Table 4.44 Pigments from Elevated pH Pea Puree Stored for 18 Months at -10°F

Band	Colour	Distance Travelled (cm)	Absorption Maxima (nm)	Solvent	Identity
E13	yellow	14.5-15.0	424,450,478	n-hexane	β-carotene
E14	pale grey	13.25-13.75	410,468,505,532, 560,610,666	diethyl ether	pheophytin a
E15	yellow	11.75-12.5	422,446,476	n-hexane	lutein
E16	trace	11.0 -11.25	Insufficient sample	-	pheophytin b
E17	blue-green (trace)	10.25-10.50	428-430,660	diethyl ether	chlorophyll a'
E18	blue-green	9.25-10.25	410,430,500,533, 576,614,660	diethyl ether	chlorophyll a
E19	green (trace)	7.25- 7.50	454,641	diethyl ether	chlorophyll b'
E20	green	6.25- 7.25	430,453,548,570, 594,642	diethyl ether	chlorophyll b
E21	yellow	4.5 - 5.5	Insufficient sample	-	-

16. Pigments from Normal pH Pea Puree

16.1 Storage Temperature 68°F

Pigment was extracted with acetone from pea puree processed at 300°F and stored for 18 months as described previously, and transferred to diethyl ether. The ether solution was chromatographed on thin layers of cellulose with solvent A, the pigment bands eluted and rechromatographed on columns and thin layers, and the pigments characterised by their absorption spectra. The absorption maxima are presented in Table 4.45. The gross

pigment spectrum is given in Graph 12.

Table 4.45 Pigments from Normal pH Pea Puree Stored for 18 Months at 68°F

Band	Colour	Distance Travelled (cm)	Absorption Maxima (nm)	Solvent	Identity
N1	yellow	14.5-15.0	424,450,478	n-hexane	β-carotene
N2	grey	12.5-13.5	410,468,505,532, 560,610,666	diethyl ether	pheophytin a
N3	yellow	11.5-12.25	422,446,476	n-hexane	lutein
N4	yellow-green	10.75-11.25	412,434,524,556, 600,653	diethyl ether	pheophytin b
N5	grey	10.0-10.50	401,664	diethyl ether	pigment X
N6	yellow	9.0- 9.25	insufficient sample	-	-
N7	yellow-green	7.75-8.25	427,653	diethyl ether	pigment Y
N8	faint yellow	4.5 -5.0	insufficient sample	-	-

16.2 Storage Temperature -10°F

Pigment was extracted with acetone from pea puree processed at 300°F and stored for 18 months, transferred to diethyl ether, and chromatographed on thin layers of cellulose. The bands were eluted and rechromatographed and the pigments characterised by their absorption spectra. Results are presented in Table 4.46.

Table 4.46 Pigments from Normal pH Pea Puree Stored for 18 Months at -10°F

Band	Colour	Distance Travelled (cm)	Absorption Maxima (nm)	Solvent	Identity
N9	yellow	14.5-15.0	424,450,478	n-hexane	β-carotene
N10	grey	12.75-13.25	410,468,505,532, 560,610,666	diethyl ether	pheophytin a
N11	yellow	11.75-12.50	422,446,476	n-hexane	lutein
N12	yellow-green	11.0-11.50	433,653 (trace)	diethyl ether	Phoephytin b
N13	faint yellow	10.5-10.75	insufficient sample	-	-
N14	blue-green	10.25-10.50	428-430,660	diethyl ether	chlorophyll a'
N15	blue-green	9.0 -10.25	410,429,500,533, 577,615,660	diethyl ether	chlorophyll a
N16	green (grace)	7.75- 8.00	454,642	diethyl ether	chlorophyll b'
N17	green	6.5- 7.75	430,453,548,569, 594,642	diethyl ether	chlorophyll b
N18	yellow	4.75- 5.0	insufficient sample	-	-

17. Characterisation of Unusual Pigments from Processed Pea Puree

17.1 Visible Spectra

Unusual chlorophyll-type pigments isolated from processed pea puree were characterised by their visible absorption spectra in diethyl ether and/or acetone. Pigments in bands E11 and E12 were present in low concentrations and could not be characterised further. The absorption maxima in ether and acetone of the major pigment fractions X, Y and Z (bands E5, E7.1 and E10 respectively) are presented in Table 4.43. The blue/red absorbance ratio in diethyl ether for preparations of pigments X, Y and Z, together with ratios for chlorophyll a and b and pheophytins a and b

are presented in Table 4.47.

Table 4.47 Absorbance Ratio $\frac{\text{blue}}{\text{red}}$ in Diethyl Ether for Chlorophyll-Derived Pigments

Pigment	Absorbance Ratio $\frac{\text{blue}}{\text{red}}$
Chlorophyll a	1.32 - 1.33
Chlorophyll b	2.81 - 2.84
Pheophytin a	2.03 - 2.08
Pheophytin b	5.16 - 5.22
Pigment X	2.84 - 2.93
Pigment Y	6.82 - 6.92
Pigment Z	4.44 - 4.51

The effect of acid on the spectra of pigments X, Y and Z was examined by taking aliquots of the pigments in acetone, adding 1 drop concentrated hydrochloric acid, and vacuum evaporating the solutions to dryness at room temperature or in a stream of oxygen-free nitrogen. Repeated addition of a small amount of acetone followed by evaporation ensured removal of moisture. The pigments were dissolved in diethyl ether and their spectra recorded. Absorption maxima of pigments X and Y for the untreated and acid-treated samples were identical, although the absorbance ratio blue/red had altered slightly. For pigment X, the ratio had increased to 3.16 while for pigment Y, the ratio had increased to 7.09. The addition of acid to pigment Z resulted in a colour change from green to a yellowish-green. The absorption spectrum of acid-treated pigment Z was very similar to that of pigment Y, showing maxima at 427 and 653nm in diethyl ether. The blue/red absorbance ratio was 6.95. Acid-treated pigment Z co-chromatographed with pigment Y on thin layers of cellulose (solvent A)

when applied together as a streak.

17.2 Infrared Spectra

Concentrated solutions of purified pigment X and pigment Y were examined in a Hilger and Watts Infrascan H900 Recording Spectrophotometer. Pigment solutions in carbon tetrachloride (spectroscopic grade) were injected by syringe into a liquid cell (R.I.I.C., F-01, light path 0.1143mm) with sodium chloride optics. The solvent was placed in a sodium chloride cell with a variable light path (R.I.I.C.) adjusted so that solvent absorption was cancelled when solvent was placed in both the sample and reference beams. The instrument was adjusted by calibration against the 1603cm^{-1} peak of maximum absorption in the spectrum of a polystyrene film. Spectra of purified pheophytins a and b were also recorded, and are shown in Graph 22.

17.3 Chemical Tests

(i) Phase test: A diethyl ether solution of the pigment was carefully underlayered with approximately the same volume of 30% w/v methanolic potassium hydroxide. In a positive test, a coloured ring was produced at the interface of the aqueous and organic phases. For the a series, a yellow ring was formed, and in the b series, a red ring was formed. After a short time, the pigment was extracted into the alkaline phase, leaving the ether colourless.

(ii) Solubility in acid: The pigment was distributed between diethyl ether and aqueous solutions of hydrochloric acid of varying concentration. The hydrochloric acid number, defined as the percent by weight of hydrochloric acid in solution which extracted more than two-thirds of the pig-

ment from an equal volume of ether, was determined.

(iii) Solubility in alkali: Diethyl ether solutions of pigments were extracted with 0.01 N potassium hydroxide. A positive test indicated solubility of the pigment in alkali, leaving a colourless ether phase. The results of tests on pigments X, Y and Z, as well as on purified chlorophylls a and b, pheophytins a and b and pheophorbides a and b are presented in Table 4.48.

Table 4.48 Chemical Tests on Chlorophylls and Their Derivatives

Pigment	Phase Test	Approximate Hydrochloric Acid Number	Solubility in 0.01N KOH
Chlorophyll a	+	-	-
Chlorophyll b	+	-	-
Pheophytin a	+	28 - 29	-
Pheophytin b	+	34 - 35	-
Pheophorbide a	+	14 - 15	+
Pheophorbide b	+	19 - 20	+
Pigment X	-	22 - 23	-
Pigment Y	-	26 - 27	-
Pigment Z	-	-	-

18. Model System Studies

18.1 Reaction of Pigments with Sodium Bicarbonate

18.11 Pheophytin a

Pheophytin a, obtained by acid conversion of purified chlorophyll a as previously described, was further purified by chromatography on thin layers of cellulose with solvent A. The principal grey band of pheophytin a was scraped off the plates, the pigment eluted with acetone, and the acetone solution diluted with aqueous sodium bicarbonate to give an $\frac{M}{100}$

sodium bicarbonate solution in 50% (v/v) acetone. The solution was incubated for 4 days in the dark at room temperature, pigment transferred to diethyl ether, the ether solution washed with distilled water, dried over anhydrous sodium sulphate and chromatographed on thin layers of cellulose with solvent **A**. Where sufficient material was available, the separated bands were rechromatographed on thin layers of cellulose with solvent **A** or solvent B, and the pigment eluted with diethyl ether. Absorption maxima are given in Table 4.49. Spectra of isolated pigments are shown in Graph 23.

Table 4.49 Pigments from Reaction of Pheophytin a with Sodium Bicarbonate

Band	Colour	Distance Travelled (cm)	Absorption Maxima (nm) (diethyl ether)	Absorbance Ratio blue/red
B1	grey	12.50-13.25	410,470,506,535,562,609,667	2.08
B2	faint grey	11.75-12.00	408,504,532,540,608,665,692	2.20
B3	grey	10.75-11.25	401,500,530,614,671	2.70
B4	faint grey	10.0 -10.25	insufficient sample	-
B5	pale grey	9.25- 9.75	400,500,529,665	3.16

18.12 Pheophytin b

Purified pheophytin b was incubated as described for pheophytin a and chromatographed on thin layers of cellulose. Bands B6, B8 and B9 were rechromatographed by cellulose TLC, and the pigments eluted with diethyl ether. Absorption maxima are given in Table 4.50. Spectra are shown in Graph 24.

Table 4.50 Pigments from Reaction of Pheophytin b with Sodium Bicarbonate

Band	Colour	Distance Travelled (cm)	Absorption Maxima (nm) (diethyl ether)	Absorbance Ratio blue/red
B6	yellow-green	11.25-11.75	412,434,524,556,600,654	5.20
B7	faint yellow	11.0 -11.25	insufficient sample	-
B8	yellow-green	10.25-10.75	427,523,557,600,653-654	5.80
B9	yellow-green	9.0 - 9.5	427,520,556,599,654-655	5.36
B10	faint yellow	7.5 - 7.75	insufficient sample	-

18.13 Chlorophyll b

Purified chlorophyll a was incubated as described above and chromatographed on thin layers of cellulose with solvent A. The bands were re-chromatographed on thin layers of cellulose and the pigments eluted with diethyl ether. Absorption maxima are presented in Table 4.51. Spectra are shown in Graph 25.

Table 4.51 Pigments from Reaction of Chlorophyll b with Sodium Bicarbonate

Band	Colour	Distance Travelled (cm)	Absorption Maxima (nm) (diethyl ether)	Absorbance Ratio blue/red
B11	yellow-green	9.25- 9.75	427,653	6.17
B12	pale green	5.0 - 5.25	445,630	5.04
B13	pale green	4.25- 4.5	451,641	4.00
B14	green	3.75- 4.0	444,630	5.50

18.2 Reaction of Pigments with Magnesium Carbonate

18.21 Pheophytin a

Chromatographically pure pheophytin a was incubated for 4 days in the dark at room temperature in $\frac{M}{100}$ magnesium carbonate in 50% acetone. The pigments were extracted into diethyl ether, dried and chromatographed

on thin layers of cellulose with solvent A. The bands were rechromatographed on thin layers of cellulose, the pigments eluted, and spectra recorded. Absorption maxima are presented in Table 4.52. Spectra are shown in Graph 26.

Table 4.52 Pigments from Reaction of Pheophytin a with Magnesium Carbonate

Band	Colour	Distance Travelled (cm)	Absorption Maxima (nm) (diethyl ether)	Absorbance Ratio blue/red
M1	grey	12.50-13.00	410,470,506,535,562,609,667	2.10
M2	faint grey	11.50-11.75	408,506,543,606,666,695	4.20
M3	grey	10.50-11.00	400,498,529,564,614,669	2.61
M4	pale grey	9.5 - 9.75	422,518,561,624,671	2.15

18.22 Pheophytin b

Chromatographically pure pheophytin b was incubated as described above and chromatographed on thin layers of cellulose. Each band was rechromatographed on cellulose, the pigments eluted with diethyl ether and spectra recorded. Absorption maxima are presented in Table 4.53.

Table 4.53 Pigments from Reaction of Pheophytin b with Magnesium Carbonate

Band	Colour	Distance Travelled (cm)	Absorption Maxima (cm) (diethyl ether)	Absorbance Ratio blue/red
M5	yellow-green	11.0 -11.5	412,434,524,556,600,654	5.24
M6	yellow-green	10.0 -10.5	427,523,557,600,653	5.91
M7	yellow-green	8.5 - 9.0	427,520,556,599,654-655	5.42
M8	yellow	7.25 - 7.5	insufficient sample	-

18.23 Chlorophyll b

Chromatographically pure chlorophyll b was incubated as described above and chromatographed on thin layers of cellulose with solvent A.

Each band was rechromatographed on thin layers of cellulose, the pigments eluted with diethyl ether, and spectra recorded. Absorption maxima are presented in Table 4.54.

Table 4.54 Pigments from Reaction of Chlorophyll b with Magnesium Carbonate

Band	Colour	Distance Travelled (cm)	Absorption Maxima (nm) (diethyl ether)	Absorbance Ratio blue/red
M9	yellow-green	9.5 -9.75	427,652	6.04
M10	green	6.5 - 7.0	453,642	2.86
M11	pale green	4.5 - 4.75	451,641	4.09
M12	green	3.0 - 3.25	445,630	5.52

PART II CHLOROPHYLLS, COLOUR AND LIPID OXIDATION IN FROZEN VEGETABLES AND MODEL SYSTEMS

A. STORAGE STUDIES ON FROZEN VEGETABLES

19. Preparation of Frozen Vegetables

(i) Peas: Thirty-eight pounds fresh hand-picked peas from the same batch as that used for experiments on processed pea puree (Part I) were used for storage trials. Fresh unblanched peas were packed into plain 401 x 411 tinplate containers. Before sealing half the cans were evacuated and flushed five times with nitrogen, while the remaining cans were evacuated and flushed five times with oxygen. The cans were blast frozen at -40°F.

The remaining fresh peas were blanched for one minute in boiling water, immediately cooled in running cold water, drained and packed into plain 401 x 411 tinplate containers. Half the cans were evacuated and

sealed in an atmosphere of nitrogen, and half in an atmosphere of oxygen. The cans were blast frozen at -40°F.

Commercially prepared frozen peas were obtained from Gordon Edgell Pty. Ltd., Rozelle, Sydney. The peas, grown at Sassafras, Tasmania, were of the same variety (i.e. Edgell Freezer) and from the same batch as the peas used in Parts I and II of this work. The peas were water blanched for 1¼ minutes at 205°F, frozen and transported to Sydney in cartons of 8 oz. polythene pouches.

(ii) Beans: Samples of commercial varieties of frozen green beans (Phaseolus vulgaris) grown at Bathurst and Brisbane, were obtained from Gordon Edgell Pty. Ltd., Rozelle. The samples grown at Bathurst, varieties Tendergreen and Pearlgreen, were blanched for 2 minutes in steam at 205°F, packed into 10oz. wax-lined cartons, frozen in a contact plate freezer and despatched to Sydney. The Brisbane samples, also varieties Tendergreen and Pearlgreen, were blanched for 3¼ minutes in steam at 210°F, frozen to -5°F in a fluidised bed freezer, packed into 4lb. polythene bags, and stored six weeks at 0°F before despatch to Sydney.

On arrival at the Kensington Laboratory, all commercial samples were divided into 2 lots and immediately placed on storage at 15°F and -10°F. Samples of peas prepared in the laboratory were placed on storage at 15°F and -10°F after initial blast freezing at -40°F.

20. Pigments of Frozen Vegetables

Chlorophyll pigments were determined spectrophotometrically using the methods of Dietrich (1958), Vernon (1960) and White et al.(1963) as described previously with some modifications. Pigments were extracted

from duplicate 10.0g samples of peas and 20.0g samples of beans taken from material on storage at the two temperatures. The samples were weighed while frozen, and chopped into small pieces to facilitate extraction of pigment with 100ml cold (-20°F) acetone and 1g magnesium carbonate powder. Further extraction and washing of the filtered pulp with 80% acetone removed all pigments, and the solution was made up to volume (250ml, acetone solution 1) with acetone, 80% acetone or water to give a final acetone concentration of 80%, allowing for water initially present in the frozen vegetable. Absorbances were measured in 80% acetone at selected wavelengths in 1cm cuvettes against 80% acetone as the blank. Analysis of pigments in diethyl ether by the method of White et al.(1963) was carried out on 100ml acetone solution (1) as previously described.

Results of storage trials on frozen peas and beans are presented in Tables 4.55 to 4.63. Pigment concentrations are expressed as µg/g material, based on moisture content of material at zero storage time.

Table 4.55 Pigment Content of Blanched Peas Sealed Under Nitrogen and Stored for 20 Months at 15°F and -10°F

Pigment	Pigment Content (μg/g)									
	Storage Temperature 15°F					Storage Temperature -10°F				
	Storage Time (Months)					Storage Time (Months)				
	0	3	8	13	20	0	3	8	13	20
C_a	100.4	96.2	96.5	82.4	85.9	100.4	106.7	103.7	100.9	103.8
C_b	40.9	38.2	35.4	37.8	33.5	40.9	41.2	41.9	39.5	38.6
Py_a	5.9	11.6	15.0	23.5	25.9	5.9	4.9	6.0	7.8	10.0
Py_b	0.0	2.7	4.7	3.3	8.4	0.0	0.0	1.8	1.2	2.9
Total C	141.3	134.5	131.9	120.2	119.4	141.3	147.9	145.5	140.4	142.4
Total Py	5.9	14.3	19.7	26.8	34.3	5.9	4.9	7.8	9.0	12.9
Total C + Py	147.2	148.8	151.6	147.0	153.7	147.2	152.8	153.3	149.4	155.3
% Conv.C→Py	4.0	9.6	13.0	18.2	22.3	4.0	3.2	5.1	6.0	8.3
% Conv. $C_a \rightarrow Py_a$	5.6	10.8	13.5	22.2	23.2	5.6	4.4	5.5	7.2	8.8
% Conv. $C_b \rightarrow Py_b$	0.0	6.6	11.7	8.0	20.0	0.0	0.0	4.1	2.9	7.0

Table 4.56 Pigment Content of Blanched Peas Sealed Under Oxygen and Stored for 20 Months at 15°F and -10°F

Pigment	Pigment Content (μg/g)									
	Storage Temperature 15°F					Storage Temperature -10°F				
	Storage Time (months)					Storage Time (Months)				
	0	3	8	13	20	0	3	8	13	20
C_a	100.4	101.3	94.3	81.4	81.7	100.4	109.1	104.4	98.0	102.3
C_b	40.9	36.2	34.4	33.1	33.4	40.9	36.4	39.5	35.9	37.1
Py_a	5.9	8.3	15.3	23.1	32.5	5.9	6.0	5.5	5.7	8.3
Py_b	0.0	4.2	5.1	5.7	7.5	0.0	3.6	1.4	0.0	3.5
Total C	141.3	137.5	128.7	114.5	115.1	141.3	145.5	143.9	133.9	139.4
Total Py	5.9	12.5	20.4	28.6	40.0	5.9	9.6	6.9	5.7	11.8
Total C + Py	147.2	150.0	149.1	143.1	155.1	147.2	155.1	150.8	139.6	151.2
% Conv.C→Py	4.0	8.3	13.7	20.0	25.8	4.0	6.2	4.6	4.2	7.8
% Conv.$C_a \rightarrow Py_a$	5.6	7.6	14.0	22.1	28.5	5.6	5.2	5.0	5.5	7.5
% Conv.$C_b \rightarrow Py_b$	0.0	10.4	12.9	14.7	18.3	0.0	9.0	3.4	0.6	8.6

Table 4.57 Pigment Content of Commercial Blanched Peas Stored for 20 Months at 15°F and -10°F

Pigment	Pigment Content (μg/g)									
	Storage Temperature 15°F					Storage Temperature -10°F				
	Storage Time (Months)					Storage Time (Months)				
	0	3	8	13	20	0	3	8	13	20
C_a	103.1	100.0	89.1	83.2	77.0	103.1	115.9	100.4	101.2	103.3
C_b	40.3	41.0	35.5	37.2	32.1	40.3	41.5	39.4	39.3	38.8
Py_a	7.2	11.6	21.9	30.0	32.6	7.2	5.7	7.1	8.6	10.3
Py_b	2.3	2.9	5.6	6.8	7.9	2.3	0.0	1.5	1.5	0.0
Total C	143.4	141.0	124.6	120.4	109.1	143.4	157.4	139.8	140.5	142.1
Total Py	9.5	14.5	27.5	36.8	40.5	9.5	5.7	8.6	10.1	10.3
Total C + Py	152.9	155.5	152.1	157.2	149.6	152.9	163.1	148.4	150.6	152.4
% Conv. C → Py	6.2	9.3	18.1	23.4	27.1	6.2	3.5	5.8	6.7	7.2
% Conv. $C_a \rightarrow Py_a$	6.5	10.4	19.7	26.5	29.7	6.5	4.7	6.6	7.8	9.8
% Conv. $C_b \rightarrow Py_b$	5.4	6.6	13.6	15.5	19.8	5.4	0.0	3.7	3.7	1.8

Table 4.58 Pigment Content of Unblanched Peas Sealed Under Nitrogen and Stored for 20 Months at 15°F and -10°F

Pigment	Pigment Content (μg/g)									
	Storage Temperature 15°F					Storage Temperature -10°F				
	Storage Time (Months)					Storage Time (Months)				
	0	3	8	13	20	0	3	8	13	20
C_a	99.0	66.2	28.3	25.2	4.2	99.0	96.4	90.3	95.9	80.7
Cd_a	0.0	6.6	13.4	5.6	4.8	0.0	0.0	0.0	4.2	3.3
C_b	37.4	32.5	20.5	11.7	6.6	37.4	36.8	33.2	35.8	31.7
Cd_b	0.0	1.1	0.0	2.4	1.7	0.0	0.0	0.0	0.0	2.2
Py_a	5.8	20.7	21.0	12.6	7.6	5.8	8.2	7.1	5.7	10.3
Po_a	0.0	2.4	22.2	42.9	50.0	0.0	0.0	1.9	0.4	3.8
Py_b	3.5	6.8	10.0	10.0	7.2	3.5	1.8	5.3	3.3	3.2
Po_b	0.0	7.5	6.4	12.2	20.4	0.0	0.0	0.0	2.8	1.7
Total C + Cd[1]	136.4	106.4	62.2	34.9	17.3	136.4	133.2	123.5	135.9	117.9
Total Py + Po[1]	9.3	37.4	59.6	77.6	85.2	9.3	10.0	14.3	11.7	19.0
Total C+Cd+Py+Po[1]	145.7	143.8	121.8	112.5	102.5	145.7	143.2	137.8	147.6	136.9
% Conv.C[2] → Py[3]	6.4	26.0	48.9	69.0	83.1	6.4	7.0	10.3	8.1	13.9
% Conv.C_a+Cd_a → Py_a+Po_a	5.3	24.1	50.9	72.7	86.4	5.5	7.8	8.2	5.7	14.4
% Conv.C_b+Cd_b → Py_b+Po_b	8.6	29.9	44.4	61.2	76.9	8.6	4.7	13.8	14.3	12.6
% Conv.C → Cd[1]	0.0	7.2	21.5	22.9	37.6	0.0	0.0	0.0	3.7	4.7
% Conv.Py → Po[1]	0.0	26.5	48.0	71.0	82.6	0.0	0.0	13.3	26.4	28.9

(1) - Includes a and b pigments
(2) - Includes chlorophylls (a + b) and chlorophyllides (a + b)
(3) - Includes pheophytins (a + b) and pheophorbides (a + b)

Table 4.59 Pigment Content of Unblanched Peas Sealed Under Oxygen and Stored for 20 Months at 15°F and -10°F

Pigment	Pigment Content (μg/g)									
	Storage Temperature 15°F					Storage Temperature -10°F				
	Storage Time (Months)					Storage Time (Months)				
	0	3	8	13	20	0	3	8	13	20
C_a	99.0	60.1	23.5	12.0	1.7	99.0	99.0	89.0	86.1	68.9
Cd_a	0.0	0.0	1.7	4.5	7.4	0.0	0.0	0.0	3.1	4.8
C_b	37.4	33.0	19.5	12.6	2.5	37.4	37.8	34.5	29.4	26.2
Cd_b	0.0	0.0	3.3	2.3	0.0	0.0	0.0	0.0	0.0	1.1
Py_a	5.8	22.6	21.3	18.0	9.7	5.8	7.5	14.6	10.4	14.0
Po_a	0.0	6.9	27.3	40.2	46.4	0.0	0.0	1.0	1.7	4.5
Py_b	3.5	10.0	4.0	6.0	5.3	3.5	3.3	5.2	3.2	3.6
Po_b	0.0	3.3	10.5	10.3	20.7	0.0	0.0	0.0	1.1	1.3
Total C + Cd	136.4	93.1	48.0	31.4	11.6	136.4	136.8	122.9	118.6	100.9
Total Py + Po	9.3	42.8	63.1	74.6	82.1	9.3	10.8	20.8	16.4	23.4
Total C+Cd+Py+Po	145.7	135.9	111.1	106.0	93.7	145.7	147.6	143.7	135.0	124.3
% Conv. C → Py	6.4	31.3	56.8	70.4	87.0	6.4	7.3	14.4	12.1	18.8
% Conv. $C_a+Cd_a \rightarrow Py_a+Po_a$	5.5	32.7	65.9	77.9	86.0	5.5	7.0	14.8	11.9	20.1
% Conv. $C_b+Cd_b \rightarrow Py_b+Po_b$	8.6	28.5	38.9	52.2	89.0	8.6	8.0	13.1	12.4	15.2
% Conv. C → Cd	0.0	0.0	10.4	21.7	65.9	0.0	0.0	0.0	3.3	5.8
% Conv. Py → Po	0.0	23.8	59.9	67.7	81.7	0.0	0.0	4.8	17.1	24.8

Table 4.60 Pigment Content of Tendergreen (Bathurst) Beans Stored for 20 Months at 15°F and -10°F

Pigment	Pigment Content (µg/g)									
	Storage Temperature 15°F					Storage Temperature -10°F				
	Storage Time (Months)					Storage Time (Months)				
	0	3	8	13	20	0	3	8	13	20
C_a	63.6	44.5	25.3	14.7	7.0	63.6	59.0	63.5	58.4	62.2
C_b	24.4	23.1	18.3	13.6	10.2	24.4	24.0	25.6	24.1	23.0
Py_a	10.2	28.3	42.5	54.1	62.2	10.2	14.8	13.3	11.7	13.6
Py_b	3.6	8.6	16.6	17.1	20.2	3.6	4.0	1.3	3.1	2.6
Total C	88.0	67.6	43.6	27.7	17.2	88.0	83.3	89.2	82.5	85.2
Total Py	13.8	36.9	59.1	71.2	82.4	13.8	18.5	14.6	14.8	16.2
Total C + Py	101.8	104.5	102.7	98.9	99.6	101.8	101.8	103.8	97.3	101.4
% Conv. $C \rightarrow Py$	13.6	35.3	57.5	72.0	82.7	13.6	18.2	14.1	15.2	16.0
% Conv. $C \rightarrow Py_a$	13.8	38.9	62.7	79.3	89.9	13.8	20.1	17.3	16.7	17.9
% Conv. $C_b^a \rightarrow Py_b$	12.9	27.1	47.6	55.7	66.4	12.9	14.3	5.0	11.4	10.2

Table 4.61 Pigment Content of Tendergreen (Brisbane) Beans Stored for 20 Months at 15°F and -10°F

Pigment	Pigment Content (µg/g)									
	Storage Temperature 15°F					Storage Temperature -10°F				
	Storage Time (Months)					Storage Time (Months)				
	0	3	8	13	20	0	3	8	13	20
C_a	46.0	31.7	14.1	8.0	4.5	46.0	45.5	46.7	44.8	45.0
C_b	21.7	17.2	13.0	9.0	8.0	21.7	22.0	20.3	21.9	21.3
Py_a	10.0	26.1	38.5	51.2	55.6	10.0	10.5	9.8	12.7	13.9
Py_b	2.0	7.1	11.4	13.4	12.7	2.0	1.0	3.8	4.0	1.6
Total C	67.7	48.9	27.1	17.0	12.5	67.7	67.5	67.0	66.7	66.3
Total Py	12.0	31.2	49.9	64.6	68.3	12.0	11.5	13.6	16.7	15.5
Total C + Py	79.7	80.1	77.0	81.6	80.8	79.7	79.0	80.6	83.4	81.8
% Conv. $C \rightarrow Py$	15.1	39.0	64.8	79.2	84.5	15.1	14.6	16.9	20.0	18.9
% Conv. $C \rightarrow Py_a$	79.9	45.2	73.2	86.5	92.5	79.9	18.8	17.3	22.1	23.6
% Conv. $C_b^a \rightarrow Py_b$	8.4	29.2	46.7	59.8	61.4	8.4	4.3	15.8	15.4	7.0

Table 4.62 Pigment Content of Pearlgreen (Bathurst) Beans Stored for 20 Months at 15°F and -10°F

Pigment	Pigment Content (µg/g)									
	Storage Temperature 15°F					Storage Temperature -10°F				
	Storage Time (Months)					Storage Time (Months)				
	0	3	8	13	20	0	3	8	13	20
C_a	42.0	32.1	17.7	8.0	4.0	42.0	39.5	42.5	41.4	40.0
C_b	16.5	12.2	9.5	8.1	6.3	16.5	17.5	14.5	15.2	16.1
Py_a	6.2	16.2	29.1	37.1	40.2	6.2	9.1	6.1	7.8	8.1
Py_b	2.3	6.2	9.5	8.8	9.6	2.3	2.0	3.7	2.6	2.1
Total C	58.5	44.3	27.2	16.1	10.3	58.5	57.0	57.0	56.6	56.1
Total Py	8.5	22.4	38.6	45.9	49.8	8.5	11.1	9.8	10.4	10.2
Total C + Py	67.0	66.7	65.8	62.0	60.1	67.0	68.1	66.8	67.0	66.3
% Conv. $C \rightarrow Py$	12.7	33.6	58.7	74.0	82.8	12.7	16.3	14.7	15.6	15.4
% Conv. $C_a \rightarrow Py_a$	12.9	33.5	62.2	82.3	91.0	12.9	18.7	12.6	15.9	16.8
% Conv. $C_b \rightarrow Py_b$	12.2	33.7	50.0	52.1	60.4	12.2	10.3	20.3	14.6	11.5

Table 4.63 Pigment Content of Pearlgreen (Brisbane) Beans Stored for 20 Months at 15°F and -10°F

Pigment	Pigment Content (µg/g)									
	Storage Temperature 15°F					Storage Temperature -10°F				
	Storage Time (Months)					Storage Time (Months)				
	0	3	8	13	20	0	3	8	13	20
C_a	45.4	33.7	22.9	16.1	2.4	45.4	44.2	45.1	42.1	36.5
C_b	19.5	12.0	10.4	6.4	6.0	19.5	18.6	19.9	20.4	20.5
Py_a	9.0	21.2	32.2	37.5	48.8	9.0	6.2	7.2	7.9	12.5
Py_b	1.7	8.0	10.6	14.4	13.9	1.7	5.0	3.1	6.1	4.3
Total C	64.9	45.7	33.3	22.5	8.4	64.9	62.8	65.0	62.5	57.0
Total Py	10.7	29.2	42.8	51.9	62.7	10.7	11.2	10.3	14.0	16.8
Total C + Py	75.6	74.9	76.1	74.4	71.1	75.6	74.0	75.3	76.5	73.8
% Conv. $C \rightarrow Py$	14.2	39.0	56.3	69.7	88.2	14.2	15.1	13.7	18.3	22.8
% Conv. $C \rightarrow Py_a$	31.6	38.6	58.4	70.0	95.3	31.6	12.3	13.8	15.8	25.5
% Conv. $C_b \rightarrow Py_b$	8.0	40.0	50.5	69.2	69.8	8.0	21.2	13.5	23.0	17.3

21. Colour Measurements on Frozen Vegetables

21.1 Method of Colour Measurement

Samples of frozen peas and beans were removed from storage, thawed and blended with distilled water to form a puree of approximately 87% final moisture content similar to that of puree used in Part I (Section A). Unblanched peas were blended in an atmosphere of nitrogen to retard enzymic oxidations. Any air incorporated during the blending was removed by deaeration under vacuum. Thawed beans were blended to give a puree of similar consistency to that of peas and then deaerated. The same quantities of frozen material and water were blended after each storage period for each material.

Duplicate Hunterlab readings were measured on pureed samples using $\frac{1}{2}$ inch deep plastic cells, after the instrument was standardised with the green tile. Subjective colour rankings were performed by a panel of judges on thawed whole and pureed samples in small glass beakers under artificial illumination.

21.2 Colour Measurement of Stored Frozen Vegetables

Hunterlab L, a and b values for frozen peas and beans stored for 20 months at 15°F and -10°F are presented in Tables 4.64 to 4.72. The derived colour indices $^{-a}/_{b}$, $^{-a}/_{L}$ and $(a^2+b^2)^{\frac{1}{2}}$ are also presented. Multiple correlation coefficients and regression equations relating percent conversion of chlorophylls (plus chlorophyllides) to pheophytins (plus pheophorbides) with colour indices $^{-a}/_{b}$, $^{-a}/_{L}$ and $(a^2+b^2)^{\frac{1}{2}}$ for frozen peas and beans were calculated by computer (IBM CDC 3600) at the C.S.I.R.O. Division of Computing Research, Canberra.

Table 4.64 Objective Colour Indices of Blanched Peas Sealed under Nitrogen and Stored for 20 Months at 15°F and -10°F

Colour Index	Storage Temperature 15°F					Storage Temperature -10°F				
	Storage Time (Months)					Storage Time (Months)				
	0	3	8	13	20	0	3	8	13	20
L	44.4	43.1	41.6	40.2	39.8	44.4	43.6	43.7	43.5	43.2
a	-19.5	-18.6	-17.4	-16.2	-15.5	-19.5	-19.4	-19.3	-19.1	-19.0
b	24.9	24.3	23.7	23.4	23.5	24.9	24.7	24.4	24.6	24.3
$-a/b$	0.783	0.765	0.734	0.692	0.660	0.783	0.785	0.791	0.776	0.782
$-a/L$	0.439	0.432	0.418	0.403	0.389	0.439	0.445	0.442	0.439	0.440
$(a^2+b^2)^{\frac{1}{2}}$	31.62	30.60	29.40	28.46	28.15	31.62	31.41	31.11	31.14	30.85

Table 4.65 Objective Colour Indices of Blanched Peas Sealed Under Oxygen and Stored for 20 Months at 15°F and -10°F

Colour Index	Storage Temperature 15°F					Storage Temperature -10°F				
	Storage Time (Months)					Storage Time (Months)				
	0	3	8	13	20	0	3	8	13	20
L	44.4	40.9	39.8	39.8	39.6	44.4	43.3	43.5	43.6	43.9
a	-19.5	-17.8	-16.6	-15.2	-14.0	-19.5	-19.3	-19.6	-19.4	-19.8
b	24.9	23.3	22.9	22.6	22.3	24.9	24.5	24.5	24.5	25.6
$-a/b$	0.783	0.764	0.725	0.672	0.627	0.783	0.788	0.800	0.792	0.773
$-a/L$	0.439	0.435	0.417	0.382	0.354	0.439	0.446	0.451	0.445	0.451
$(a^2+b^2)^{\frac{1}{2}}$	31.62	29.32	28.28	27.23	26.33	31.62	31.19	31.38	31.25	32.36

Table 4.66 Objective Colour Indices of Commercial Blanched Peas Stored for 20 Months at 15°F and -10°F

Colour Index	Storage Temperature 15°F					Storage Temperature -10°F				
	Storage Time (Months)					Storage Time (Months)				
	0	3	8	13	20	0	3	8	13	20
L	44.2	42.8	42.3	40.6	42.2	44.2	43.1	43.4	43.0	44.0
a	-19.7	-18.1	-17.7	-15.6	-15.2	-19.7	-18.9	-19.5	-18.8	-19.4
b	25.1	24.1	24.1	23.0	24.4	25.1	24.4	24.4	24.2	25.4
$-a/b$	0.785	0.751	0.734	0.678	0.623	0.785	0.775	0.800	0.777	0.764
$-a/L$	0.446	0.423	0.418	0.384	0.360	0.446	0.439	0.449	0.437	0.441
$(a^2+b^2)^{\frac{1}{2}}$	31.90	30.14	29.90	27.79	28.75	31.90	30.86	31.23	30.64	31.97

Table 4.67 Objective Colour Indices of Unblanched Peas Sealed Under Nitrogen and Stored for 20 Months at 15°F and -10°F

Colour Index	Storage Temperature 15°F					Storage Temperature -10°F				
	Storage Time (Months)					Storage Time (Months)				
	0	3	8	13	20	0	3	8	13	20
L	43.3	43.5	46.5	47.4	49.0	43.3	45.0	41.0	41.5	42.5
a	-15.9	-11.1	-7.2	-5.7	-5.2	-15.9	-14.8	-14.5	-14.7	-14.5
b	23.3	21.0	18.6	19.2	19.3	23.3	23.0	21.0	21.3	21.6
$-a/b$	0.682	0.529	0.387	0.297	0.269	0.682	0.643	0.690	0.690	0.671
$-a/L$	0.367	0.255	0.155	0.120	0.106	0.367	0.329	0.354	0.354	0.341
$(a^2+b^2)^{\frac{1}{2}}$	28.21	23.75	19.94	20.02	19.98	28.21	27.35	25.52	25.89	26.02

Table 4.68 Objective Colour Indices of Unblanched Peas Sealed Under Oxygen and Stored for 20 Months at 15°F and -10°F

Colour Index	Storage Temperature 15°F					Storage Temperature -10°F				
	Storage Time (Months)					Storage Time (Months)				
	0	3	8	13	20	0	3	8	13	20
L	43.3	47.6	45.2	48.9	48.2	43.3	45.3	40.9	40.8	41.9
a	-15.9	-11.3	-6.3	-6.0	-4.1	-15.9	-15.4	-14.0	-14.7	-14.6
b	23.3	21.1	17.8	20.3	16.9	23.3	23.0	20.9	20.4	20.2
$-a/b$	0.682	0.536	0.354	0.296	0.243	0.682	0.670	0.670	0.721	0.723
$-a/L$	0.367	0.237	0.139	0.123	0.085	0.367	0.340	0.342	0.360	0.348
$(a^2+b^2)^{\frac{1}{2}}$	28.21	23.94	18.88	21.17	17.39	28.21	27.68	25.15	25.15	24.92

Table 4.69 Objective Colour Indices of Tendergreen (Bathurst) Beans Stored for 20 Months at 15°F and -10°F

Colour Index	Storage Temperature 15°F					Storage Temperature -10°F				
	Storage Time (Months)					Storage Time (Months)				
	0	3	8	13	20	0	3	8	13	20
L	37.9	31.8	34.1	36.1	36.9	37.9	35.0	31.7	37.9	36.6
a	-13.5	-9.0	-7.3	-5.5	-4.5	-13.5	-13.0	-10.7	-14.0	-13.2
b	18.8	15.4	15.7	16.7	16.3	18.8	17.8	14.5	19.9	18.9
$-a/b$	0.718	0.584	0.465	0.329	0.276	0.718	0.730	0.738	0.704	0.698
$-a/L$	0.356	0.283	0.214	0.152	0.122	0.356	0.371	0.338	0.369	0.361
$(a^2+b^2)^{\frac{1}{2}}$	23.15	17.84	17.31	17.58	16.91	23.15	22.04	18.02	24.33	23.05

Table 4.70 Objective Colour Indices of Tendergreen (Brisbane) Beans Stored for 20 Months at 15°F and -10°F

Colour Index	Storage Temperature 15°F					Storage Temperature -10°F				
	Storage Time (Months)					Storage Time (Months)				
	0	3	8	13	20	0	3	8	13	20
L	37.3	37.6	33.7	38.1	40.9	37.3	35.4	37.5	38.6	39.2
a	-13.7	-8.1	-5.0	-4.4	-4.4	-13.7	-12.0	-12.8	-13.5	-14.2
b	19.1	18.5	14.7	16.9	17.8	19.1	17.7	19.0	20.1	21.0
$-a/b$	0.717	0.438	0.340	0.260	0.247	0.717	0.678	0.674	0.672	0.676
$-a/L$	0.367	0.215	0.148	0.115	0.108	0.367	0.339	0.341	0.350	0.362
$(a^2+b^2)^{\frac{1}{2}}$	23.50	20.19	15.52	17.46	18.34	23.50	21.39	22.91	24.22	25.35

Table 4.71 Objective Colour Indices of Pearlgreen (Bathurst) Beans Stored for 20 Months at 15°F and -10°F

Colour Index	Storage Temperature 15°F					Storage Temperature -10°F				
	Storage Time (Months)					Storage Time (Months)				
	0	3	8	13	20	0	3	8	13	20
L	37.4	35.8	33.1	37.7	40.5	37.4	38.3	36.1	38.7	39.1
a	-14.0	-9.2	-5.8	-5.3	-4.5	-14.0	-14.3	-13.4	-14.5	-14.5
b	19.2	17.7	14.4	15.6	15.9	19.2	19.6	18.0	20.3	20.7
$-a/b$	0.729	0.520	0.403	0.340	0.283	0.729	0.730	0.744	0.714	0.700
$-a/L$	0.374	0.257	0.175	0.141	0.111	0.374	0.373	0.371	0.375	0.371
$(a^2+b^2)^{\frac{1}{2}}$	23.76	19.94	15.52	16.48	16.52	23.76	24.26	22.44	24.95	25.27

Table 4.72 Objective Colour Indices of Pearlgreen (Brisbane) Beans Stored for 20 Months at 15°F and -10°F

Colour Index	Storage Temperature 15°F					Storage Temperature -10°F				
	Storage Time (Months)					Storage Time (Months)				
	0	3	8	13	20	0	3	8	13	20
L	38.9	35.2	36.2	39.6	39.4	38.9	37.6	37.0	41.0	41.9
a	-14.7	-6.2	-4.5	-4.6	-4.0	-14.7	-13.7	-12.6	-14.0	-14.8
b	20.7	16.8	15.2	17.1	16.8	20.7	20.4	19.5	21.8	22.9
$-a/b$	0.710	0.369	0.296	0.269	0.238	0.710	0.672	0.646	0.642	0.646
$-a/L$	0.378	0.176	0.124	0.116	0.102	0.378	0.364	0.341	0.341	0.353
$(a^2+b^2)^{\frac{1}{2}}$	25.40	17.91	15.85	17.71	17.27	25.40	24.58	23.22	25.90	27.27

22. Lipid Oxidation in Frozen Vegetables

22.1 2-Thiobarbituric Acid Method

22.11 Calibration for Malonaldehyde

1,1,3,3-tetraethoxypropane, TEP (Reagent Grade) was redistilled twice in a Quickfit microdistillation outfit, and the clear distillate boiling at approximately 212°C collected. Purified TEP (0.1102g) was dissolved in 200ml distilled water and 0.5ml 1N hydrochloric acid added. The solution was heated in a water bath for one hour at 50°C in a tightly stoppered volumetric flask, cooled to 20°C, and made to volume (500ml) with distilled water. The standard malonaldehyde (MA) solution formed (10^{-3}M) was diluted 1:50 with distilled water and aliquots of 1.0 to 5.0ml (containing 2 x 10^{-8} to 10 x 10^{-8} moles malonaldehyde/5ml) transferred by pipette into clean test tubes using E-mil Greenline calibrated glassware. The aliquots were made up to 5.0ml with distilled water, 5.0ml 0.02M 2-thiobarbituric acid (TBA) added (0.228g/100ml water, A.R. grade), the mixture shaken, and the tubes, stoppered with aluminium bacteriological caps, placed in a boiling water bath for 40 minutes. Blank tubes containing 5.0ml TBA and 5.0ml distilled water were also prepared. The tubes were immediately cooled in running water, tempered to 68°F in a constant temperature water bath and the absorbances measured in 1cm cuvettes at 532nm and 600nm. The results are presented in Table 4.73 and Shown in Graph 30. The spectrum of the malonaldehyde-thiobarbituric acid complex is shown in Graph 29.

Table 4.73 Calibration for Estimation of Malonaldehyde with 2-Thiobarbituric Acid

Malonaldehyde Concentration in 5ml (moles x10^{-8})	Corrected Absorbance at 532nm	
	Readings	Mean
1	0.161, 0.154, 0.158, 0.155	0.157
2	0.311, 0.314, 0.313, 0.316	0.314
3	0.472, 0.471, 0.475, 0.471	0.472
4	0.619, 0.623, 0.622, 0.619	0.621
5	0.778, 0.782, 0.785, 0.779	0.781
6	0.935, 0.930, 0.930, 0.034	0.932
7	1.075, 1.084, 1.080, 1,087	1.082
8	1.232, 1.240, 1.229, 1.235	1.234
9	1.385, 1.382, 1.397, 1.390	1.389
10	1.545, 1.560, 1.550, 1.555	1.554

The mean values give a straight-line relationship when graphed against malonaldehyde concentration such that:

malonaldehyde concentration (moles x10^{-8} in 5ml)= absorbance reading x6.452.

22.12 Recovery of Malonaldehyde

The standard malonaldehyde solution (10^{-3}M) was diluted 1:50 and 1:200 with distilled water, and 50ml aliquots transferred, with 50ml distilled water, to 250ml Kjeldahl distillation flasks. The pH was adjusted to 1.5-1.6 by the addition of 1.3-1.4ml 1:2 hydrochloric acid (approximately 3.3N), porous pot and antifoam were added, and 50ml samples distilled from each flask in 10 minutes using a bunsen flame and double-wall water-cooled condenser. The distillate was thoroughly mixed, and colour developed in 5.0ml aliquots heated for 40 minutes in boiling water with 5.0ml 0.02M 2-thiobarbituric acid. Absorbances were measured at 532nm and 600nm. Malonaldehyde was also estimated as above in 5.0ml

aliquots of dilutedmalonaldehyde solutions before distillation. Results are presented in Table 4.74.

Table 4.74 Effect of Distillation on Recovery of Malonaldehyde from Aqueous Solution

Test No.	Sample	Corrected Absorbances at 532nm	Mean	% Recovery
1	Before distillation	0.392,0.386,0.386,0.398,0.389, 0.386	0.389	69.33
	After distillation	0.273,0.274,0.268,0.269,0.272, 0.268	0.270	
2	Before distillation	1.500,1.515,1.520,1.510,1.510, 1.510	1.511	69.61
	After distillation	1.062,1.040,1.055,1.055,1.050, 1.055	1.053	

Calculation of Distillation Constant and TBA Number

The distillation constant K, required to convert absorbance readings on distillates from oxidised materials into thiobarbituric acid (TBA) numbers (defined as mg malonaldehyde MA/1000g sample) is given by the expression

$$K\ (\text{distillation}) = \frac{\text{moles MA/5ml distillate x Mol.Wt MA x } 10^7 \text{ x } 10^2}{\text{corrected absorbance at 532nmxwt sample x\% recovery}}$$

Hence for a 20g sample and average % recovery of 69.47% (Table 4.74)

$$K\ (\text{distillation}) = \frac{4 \times 10^{-8} \times 72 \times 10^7 \times 10^2}{0.621 \times 20 \times 69.47} = 3.338$$

where 0.621 is the average absorbance of a solution containing 4×10^{-8} moles MA/5ml (Table 4.73)

$\therefore$ TBA number (mg MA/1000g sample) = absorbance x 3.338

22.13 Determination of TBA Number of Frozen Vegetables

The method of determination of the TBA number of peas and beans during frozen storage was a modification of the distillation method of Tarladgis et al.(1960). Duplicate 20.0g samples frozen peas or beans

were macerated quickly with pestle and mortar and blended thoroughly for 3 minutes at high speed in an Atomix blender with 60ml solution containing distilled water and sufficient 1:2 hydrochloric acid (3.3N) to give a slurry pH of 1.5-1.6. The slurry was quantitatively transferred to a 250ml Kjeldahl distillation flask using a further 40ml distilled water. After addition of porous pot and 2 drops of antifoam solution, 50ml aqueous solution was distilled into a volumetric flask in 10 $\pm$ 0.5 minutes using a bunsen flame. Duplicate 5.0ml aliquots distillate were transferred to test tubes, together with 5.0ml freshly prepared 0.02M TBA solution. The contents were mixed, and the covered tubes heated 40 minutes in a boiling water bath. The absorbance of the pink solutions was measured at 532nm and 600nm against a blank prepared from distilled water and TBA solution. Solutions (100ml) containing water, acid and antifoam were also distilled as a blank. In all cases the corrected absorbance of these solutions, after treatment with TBA, did not exceed 0.005 units. TBA numbers (mg malonaldehyde/1000g sample) determined on stored frozen peas and beans are presente d in Table 4.75 and are shown in Graph 32.

Table 4.75 TBA Numbers of Frozen Peas and Beans Stored for 20 Months at 15°F and -10°F

Material	TBA Numbers (mg MA/1000g)									
	Storage Temperature 15°F					Storage Temperature -10°F				
	Storage Time (Months)					Storage Time (Months)				
	0	3	8	13	20	0	3	8	13	20
Blanched, N_2	0.12	0.13	0.16	0.18	0.21	0.12	0.14	0.17	0.18	0.19
Blanched, O_2	0.12	0.14	0.19	0.24	0.25	0.12	0.15	0.16	0.20	0.23
Commercial	0.17	0.18	0.23	0.23	0.26	0.17	0.16	0.18	0.19	0.22
Unblanched, N_2	0.21	0.42	0.59	0.87	1.02	0.21	0.31	0.48	0.64	0.70
Unblanched, O_2	0.21	0.49	0.82	1.22	1.60	0.21	0.44	0.56	0.72	1.10
TG[1], BH[3]	0.16	0.17	0.18	0.23	0.26	0.16	0.15	0.14	0.22	0.24
TG , BB[4]	0.15	0.18	0.24	0.30	0.32	0.15	0.19	0.17	0.20	0.22
PG[2], BH	0.18	0.27	0.46	0.69	0.91	0.18	0.21	0.30	0.34	0.36
PG , BB	0.20	0.34	0.58	0.90	1.12	0.20	0.20	0.19	0.36	0.39

(1) Tendergreen variety

(2) Pearlgreen variety

(3) Grown at Bathurst

(4) Grown at Brisbane

22.2 Ferric Thiocyanate Method

22.21 Extraction of Lipids from Frozen Vegetables

Lipid material was extracted from frozen vegetables by a modification of the method of Bligh and Dyer (1959) as used by Walker (1964a).

(i) Peas: 50g frozen peas (moisture content 75%)were chopped finely, and blended for 3 minutes at high speed with a mixture of 50ml chloroform (A.R. grade), 100ml acidified (0.04N HCl) methanol, and 2.5ml water, the organic solvents being kept at -20°F. To the mixture was added 50ml chloroform then 50ml distilled water and the solution blended for 30 seconds after each addition of solvent. The homogenate was centrifuged for 10 minutes at 3000rpm and three fractions separated: (1) upper cloudy aqueous methanol layer, (2) plug of cell debris, (3) lower clear green chloroform layer containing lipid material. The upper methanol layer was decanted, and the lower chloroform layers vacuum filtered through sintered glass. The pulp was extracted with about 50ml chloroform, the slurry filtered, and extracted with a further portion of chloroform. The combined chloroform extracts were transferred, after filtration through Whatman No.41 paper on a buchner funnel, to a 250ml graduated cylinder, and any residual methanol allowed to separate from the green chloroform layer. The methanol layer was removed by aspiration, and the total volume of chloroform recorded.

(ii) Beans: 50g chopped frozen beans (moisture content approx. 90%) were blended with a mixture of 56ml chloroform and 112ml acidified methanol. To the mixture was added, separately with blending, 56ml chloroform and 56ml distilled water, and the slurry centrifuged. The

solutions were then treated as described for frozen peas.

22.22 Determination of Lipid Content of Chloroform Extracts

The lipid content of chloroform extracts was determined by vacuum evaporation of 50ml aliquots in tared 100ml Quickfit round bottom flasks. The flasks were stored overnight in a desiccator over phosphorus pentoxide, and the lipid content determined by increase in weight.

22.23 Calibration for Ferric Iron

0.2500g fine iron wire (99.5%, A.R. grade) cleaned with fine emery paper and wiped with tissue paper, was dissolved in 50ml 10N hydrochloric acid and 100ml distilled water, and the solution heated on a boiling water bath. One ml 100 volume (30% w/v) hydrogen peroxide (A.R. grade) was added to oxidise the iron to the ferric state, and excess peroxide removed by boiling. The solution was made to volume (250ml, 68°F) with distilled water using calibrated glassware, to give a solution containing 1mg Fe^{3+}/ml. The stock solution of ferric iron was diluted with redistilled absolute ethanol to give solutions containing 5 and 20µg Fe^{3+}/ml.

Aliquots of ethanol solutions containing 2.5 to 20µg Fe^{3+} were transferred to clean test tubes, and the volume made to 5.0ml with absolute ethanol. One ml chloroform was added, followed by 0.2ml 1:1 (5N) hydrochloric acid, 0.1ml distilled water and 1.0ml 20% (w/v) ammonium thiocyanate (A.R. grade). The absorbance of the red ferric thiocyanate complex after 3 minutes incubation was measured in 1cm cuvettes at 480nm against a blank containing the same quantities of alcohol, chloroform, acid and thiocyanate, but no added ferric iron. The results are presented in Table 4.76. The calibration curve is shown in Graph 31.

Table 4.76 Calibration for Estimation of Ferric Iron by Thiocyanate

Ferric Iron Concentration (µg in 7.3ml total volume)	Absorbance at 480nm	
	Readings	Mean
2.5	0.094, 0.092, 0.096, 0.093	0.094
5.0	0.188, 0.190, 0.188, 0.191	0.189
7.5	0.279, 0.282, 0.286, 0.283	0.283
10.0	0.374, 0.373, 0.370, 0.373	0.373
12.5	0.469, 0.465, 0.470, 0.465	0.467
15.0	0.560, 0.555, 0.557, 0.558	0.556
17.5	0.647, 0.652, 0.656, 0.649	0.651
20.0	0.748, 0.742, 0.752, 0.750	0.748

The mean values give a straight line relationship when graphed against the ferric iron concentration such that:

$$\left.\begin{array}{l}\text{Ferric iron concentration}\\(\mu\text{g in 7.3ml total volume})\end{array}\right\} = \text{absorbance reading} \times 26.81$$

22.24 Determination of Peroxides in Frozen Vegetables

One ml aliquots of acid chloroform lipid extract from frozen peas or beans (as original, concentrated or diluted solution) were added to 5.0ml absolute ethanol and 0.2ml 1:1 hydrochloric acid in clean test tubes. One tenth (0.1ml) freshly prepared 1% (w/v) ferrous ammonium sulphate (A.R. grade) was added from a calibrated syringe and 30 seconds later, 1.0ml 20% ammonium thiocyanate. The absorbance at 480nm was measured exactly 3 minutes after the addition of thiocyanate. Blank solutions were prepared containing (1) ferrous iron and thiocyanate but no chloroform extract, (2) chloroform extract and thiocyanate, but no ferrous iron, and absorbances determined as for the test solutions, to

allow for substances absorbing at 480nm other than the ferric-thiocyanate complex (e.g. pheophytins in the acid chloroform extract.

Calculation of Peroxide Value

By extrapolation of the standard calibration curve for Fe^{3+}, an absorbance of 1.0 unit corresponds to a concentration of 26.81 μg Fe^{3+} in total volume. Since 1 μ mole peroxide oxygen = 32μg peroxide oxygen $\equiv$ 112μg Fe^{3+},

$\therefore$ an absorbance of 1.0 is equivalent to $\frac{26.81}{112}$ = 0.2394 μmoles peroxide oxygen in 1ml chloroform extract. Since 1 μequivalent peroxide oxygen = 0.5 μmoles peroxide oxygen,

$\therefore$ μequivalents peroxide/ml chloroform extract = absorbance x 0.4788

For a 50g sample:

Peroxide Value (μequiv.peroxide/100g material) = absorbance x0.4788x2xV
= absorbance x0.9576xV

and Peroxide Value (μequiv.peroxide/g lipid) = $\frac{\text{absorbance x0.9576xV}}{\text{lipid content (g/100g)}}$

Peroxide values of stored frozen vegetables are presented in Table 4.77 and are shown in Graph 33.

23. pH of Frozen Vegetables

pH was determined on purees prepared for colour measurement with a Pye Dynacap pH meter calibrated with buffer solutions of known pH. The purees were diluted with distilled water. Results are presented in Table 4.78 (page 272).

Table 4.77 Peroxide Values of Lipid Extracts of Frozen Peas and Beans Stored for 20 Months at 15°F and -10°F

Peroxide Values (μeq./100g)

Material	Storage Temperature 15°F					Storage Temperature -10°F				
	Storage Time (Months)					Storage Time (Months)				
	0	3	8	13	20	0	3	8	13	20
Blanched, N_2	8.8	12.6	9.4	11.4	15.0	8.8	11.8	13.6	9.9	12.2
Blanched, O_2	8.8	13.2	11.8	13.2	17.3	8.8	6.2	9.7	19.1	12.3
Commercial	6.2	12.7	13.0	14.7	13.3	6.2	8.6	10.0	9.0	11.2
Unblanched, N_2	14.3	47.4	122.6	114.5	117.7	14.3	29.2	38.6	48.2	44.7
Unblanched, O_2	14.3	63.0	168.9	172.5	160.1	14.3	44.7	65.1	45.6	41.4
TG, BH	9.6	11.1	12.2	15.9	17.9	9.6	10.3	10.4	13.4	14.3
TG, BB	10.9	13.7	18.7	19.0	24.5	10.9	13.5	16.4	14.4	15.9
PG, BH	6.4	7.3	12.7	16.4	19.6	6.4	5.0	8.6	7.7	9.9
PG, BB	7.7	14.2	18.9	27.6	28.9	7.7	8.3	6.5	12.1	18.7

Peroxide Values (μeq./g lipid)

Material	Storage Temperature 15°F					Storage Temperature -10°F				
	Storage Time (Months)					Storage Time (Months)				
	0	3	8	13	20	0	3	8	13	20
Blanched, N_2	7.2	10.1	8.2	10.1	12.0	7.2	10.1	11.7	8.1	9.8
Blanched, O_2	7.2	11.2	10.3	11.2	15.0	7.2	4.9	8.0	15.4	9.9
Commercial	5.1	10.2	10.5	11.9	11.0	5.1	7.0	8.1	7.6	9.6
Unblanched, N_2	11.9	37.7	101.6	93.5	97.8	11.9	25.4	31.7	41.1	35.3
Unblanched, O_2	11.9	49.1	141.7	144.6	135.8	11.9	35.6	54.2	37.4	33.1
TG, BH	27.8	33.3	36.0	47.7	53.0	27.8	31.5	31.2	39.5	43.8
TG, BB	33.4	41.4	54.6	59.1	71.7	33.4	39.1	50.7	44.9	46.5
PG, BH	20.1	23.2	41.4	52.9	62.4	20.1	15.9	26.7	25.4	30.0
PG, BB	19.7	38.4	53.6	77.2	79.0	19.7	22.8	17.5	33.1	49.8

Table 4.78 pH of Frozen Peas and Beans Stored for 20 Months at 15°F and -10°F

Material	pH									
	Storage Temperature 15°F					Storage Temperature -10°F				
	Storage Time (Months)					Storage Time (Months)				
	0	3	8	13	20	0	3	8	13	20
Blanched, N_2	6.88	7.03	7.05	7.01	6.96	6.88	7.01	7.14	6.99	7.05
Blanched, O_2	6.88	7.00	7.05	7.03	7.00	6.88	7.11	7.08	6.99	7.09
Commercial	6.81	7.09	6.93	7.00	6.86	6.81	7.10	6.97	6.94	7.10
Unblanched, N_2	6.45	6.40	6.43	6.36	6.30	6.45	6.57	6.53	6.59	6.44
Unblanched, O_2	6.45	6.43	6.39	6.42	6.31	6.45	6.43	6.56	6.52	6.40
TG, BH	6.15	6.25	6.17	6.08	6.07	6.15	6.21	6.15	6.20	6.24
TG, BB	6.17	6.14	6.07	6.06	5.98	6.17	6.20	6.08	6.14	6.21
PG, BH	6.00	6.07	5.97	5.97	5.93	6.00	6.04	6.02	6.04	6.07
PG, BB	6.06	6.06	6.01	6.02	5.93	6.06	6.11	6.09	6.05	6.19

PART II B. CHLOROPHYLL BLEACHING AND LIPID OXIDATION IN MODEL SYSTEMS

24. Method for Determining Chlorophyll Bleaching Activity in Enzyme Extracts

Preliminary experiments were carried out to determine the presence in enzyme solutions of activity resulting in the loss of green colour (bleaching) when chlorophylls were incubated in model systems with unsaturated fatty acids and enzyme extracts. Model systems used throughout this work contained:

(i) Buffer: 0.2M acetate (pH4.5, 5.0, 5.5), 0.1M phosphate (pH6.0, 6.5, 7.0, 7.5, 8.0) or 0.1M borate (pH9.0) buffer solutions were prepared from A.R. grade chemicals and distilled water.

(ii) Chlorophyll: (a) Crude chlorophyll or pheophytin. Crude pigment solutions were prepared by extracting shredded, blanched spinach leaves with acetone, or methanol plus petroleum ether as previously described. The extract was evaporated to dryness under vacuum and the residue dissolved in acetone (= crude chlorophyll solution). Crude pheophytin solution was prepared from crude chlorophyll by the addition of concentrated hydrochloric acid to an aliquot of acetone solution.

(b) Purified chlorophyll or pheophytin. Pure pigment solutions were prepared by column and thin-layer chromatography of crude pigment extracts as previously described. The purified pigments were dissolved in acetone.

(iii) Fatty Acid: Purified fatty acids were obtained either from the Hormel Foundation, U.S.A. or Koch-Light Laboratories, England. The acids

were dissolved in absolute ethanol as 1% solution (= 10mg/ml) and were stored in the dark at -20°F when not in use.

(iv) Enzyme Extract: Extracts were prepared from soya bean meal or fresh or frozen vegetables by extracting ground materials with buffer solutions of known pH. Enzyme solutions were kept in ice.

Bleaching activity in enzyme extracts was usually determined by adding 22.0ml buffer, 2.0ml acetone solution of crude chlorophyll, 0.5ml ethanol solution of fatty acid and 0.5ml buffer solution of enzyme, to a 50ml stoppered volumetric flask covered with aluminium foil or black polythene. The flask was continually shaken on a Dynamax flask shaker, and 2ml aliquots of the incubated solution, withdrawn by pipette at various reaction times, were added to 8ml acetone and the 80% acetone solutions centrifuged for 5 minutes at 3000rpm to remove precipitated protein. Absorbances (A) of the clarified pigment solutions were measured at the wavelength of maximum absorption (662nm for crude chlorophyll, corrected for turbidity at 700nm) in 1cm cuvettes against a blank of 80% acetone. Corrected absorbances (A_o) of control solutions in which buffer, chlorophyll, fatty acid and heat-inactivated enzyme were incubated for the same time as the test solutions were also determined. Bleaching activity was expressed as: Percent chlorophyll bleaching = $\frac{A_o - A}{A_o} \times 100$

2.0ml crude chlorophyll solution in 25.0ml total volume after dilution of 2.0ml aliquots of incubated solution to 10.0ml with 8.0ml acetone, gave an absorbance in unbleached preparations of 0.75-0.80 at 662nm.

25. Effect of pH on Chlorophyll Bleaching by Soya Extract

To a series of clean 50ml volumetric flaskswas added 22ml buffer (pH4.5 to 9.0), 2ml crude acetone solution of chlorophyll, 0.5ml (5mg) linoleic acid (99.9%, lot 10-P, Hormel Foundation) in absolute ethanol, or 0.5ml ethanol, and 0.5ml soya enzyme solution. The soya extract was prepared from 10g powdered soya bean meal soaked 30 minutes in 50ml distilled water, centrifuged 10 minutes at 3000rpm, and the supernatant decanted and stored in ice.. The flasks were incubated in duplicate for 2 and 4 minutes, and the absorbances of diluted aliquots measured at 662nm and 700nm against a blank of 80% acetone. Results are presented in Table 4.79. Spectra of bleached chlorophyll solutions at pH6.5 are shown in Graph 34. The pH profile is shown in Graph 37.

Table 4.79 Effect of pH on Chlorophyll Bleachingby Soya Enzyme

Buffer pH	Percent Chlorophyll Bleaching			
	No Linoleic Acid		With Linoleic Acid	
	2 mins.	4 mins	2 mins	4 mins
4.50	2.1	4.5	21.8	38.6
5.01	6.9	14.8	49.5	64.3
5.57	14.3	24.6	68.7	78.1
5.99	21.7	32.3	75.5	85.9
6.53	23.0	32.1	78.9	89.4
7.00	18.8	26.7	75.6	86.3
8.05	9.7	17.1	59.6	70.9
9.03	3.7	9.8	14.5	26.0

26. Effect of Pure Lipoxygenase on Chlorophyll Bleaching by Soya Extract

20mg pure lipoxygenase enzyme (salt free lyophilised powder, ex soybean, Koch-Light Laboratories, Batch 22474, activity 80,000 units/mg)

was dissolved in 20ml pH6.5 phosphate buffer, and stored in ice. Crude soya enzyme was prepared by extracting 10g soyabean meal with 50ml pH6.5 phosphate buffer and centrifuging 10 minutes at 3000rpm. A series of flasks were prepared in duplicate containing volumes of solutions as shown below. The total volume was 25.0ml. Incubation times were 2 and 4 minutes. The results are presented in Table 4.80. Spectra are shown in Graph 35.

Contents	Con-trol	Flask Number 1	2	3	4	5	6	7
pH6.5 buffer	22.5	22.3	21.3	21.3	20.3	22.3	21.5	20.5
Crude chlorophyll (acetone)	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Ethanol	-	0.5	0.5	-	-	-	-	-
Linoleic acid solution (10mg/ml ethanol)	0.5	-	-	0.5	0.5	0.5	0.5	0.5
Soya enzyme extract	-	0.2	0.2	0.2	0.2	0.2	-	-
Pure lipoxygenase solution (mg/ml)	-	-	1.0	1.0	2.0	-	1.0	2.0

Table 4.80 Effect of Pure Lipoxygenase on Chlorophyll Bleaching by Soya Extract

Flask No.	Contents	Chlorophyll Bleaching 2 mins.	4 mins.
1	0.2ml extract	6.4	14.7
2	0.2ml extract + 1mg lipoxygenase	8.9	20.5
3	0.2ml extract + 5mg linoleic acid + 1mg lipoxygenase	81.6	93.8
4	0.2ml extract + 5mg linoleic acid + 2mg lipoxygenase	87.6	95.1
5	0.2ml extract + 5mg linoleic acid	54.4	80.2
6	5mg linoleic acid + 1mg lipoxygenase	1.2	3.8
7	5mg linoleic acid + 2mg lipoxygenase	3.4	7.2

27. Effect of Long-Chain Fatty Acids on Chlorophyll Bleaching by Soya Extract

One percent w/v ethanol solutions of long-chain fatty acids were prepared and 0.5ml aliquots (5mg) added to a series of duplicate flasks containing 22ml pH6.5 phosphate buffer, 2ml crude chlorophyll in acetone, and 0.5ml soya enzyme solution (5ml pH6.5 buffer/g soyabean meal). The fatty acids used were:

Stearic acid,C18, saturated, - Koch-Light (>95%).

Elaidic acid, C18, 1 *trans* double bond, - Koch-Light (>95%).

Oleic acid, C18, 1 *cis* double bond, - Hormel Foundation, Lot 10-P (>99%)

Linoleic acid, C18,2 *cis* double bonds, -Hormel Foundation,Lot 10-P (>99.9%).

Linolenic acid,C18,3 *cis* double bonds, -Hormel Foundation,Lot 3-P (>99.8%).

Arachidonic acid,C20,4 *cis* double bonds, -Hormel Foundation,Lot2-P(>90%).

Results are presented in Table 4.81.

Table 4.81 Effect of Different Fatty Acids on Chlorophyll Bleaching by Soya Extract

Fatty Acid (5mg)	Percent Chlorophyll Bleaching 2 mins.	4 mins.
None	12.0	19.7
Stearic	26.2	33.5
Elaidic	24.0	44.6
Oleic	25.4	48.3
Linoleic	76.3	87.7
Linolenic	81.4	90.1
Arachidonic	67.8	80.0

28. Chlorophyll Bleaching with Crude and Purified Pigments

The bleaching of solutions containing crude chlorophyll, crude pheophytin, and purified chlorophyll a and chlorophyll b was examined. Crude pheophytin solution was prepared from crude acetone solution of chlorophyll by the addition of 2 drops of hydrochloric acid. Purified chlorophyll a and chlorophyll b were prepared by column and thin-layer chromatography as previously described. Flasks were prepared containing 19ml pH6.5 phosphate buffer, 5ml acetone solution pigment (for crude chlorophyll, 2ml acetone solution chlorophyll plus 3ml pure acetone), 0.5ml linoleic acid in ethanol, and 0.5ml enzyme solution (5ml pH6.5 buffer/g soyabean meal). Incubation and sampling was as previously described. Absorbances were measured at 700nm and 662nm (crude chlorophyll), 665nm (crude pheophytin), 662nm (pure chlorophyll a) and 644nm (pure chlorophyll b). Results are presented in Table 4.82. The spectra of bleached crude pheophytin are shown in Graph 36.

Table 4.82 Bleaching of Crude and Pure Chlorophyll Pigments by Soya Enzyme

Pigment	Percent Chlorophyll Bleaching			
	No Linoleic Acid		With Linoleic Acid	
	2 mins.	4 mins.	2 mins.	4 mins
Crude Chlorophyll	16.0	21.6	50.4	74.4
Crude Pheophytin	12.0	19.7	30.8	55.6
Pure Chlorophyll a	8.5	16.0	17.5	26.6
Pure Chlorophyll b	6.7	13.3	8.4	15.3

29. Chlorophyll Bleaching Activity in Pea Extracts

Chlorophyll bleaching activity was determined in extracts from (i) fresh peas, (ii) an acetone powder prepared from fresh peas, (iii) pea seeds.

(i) Fresh market peas were shelled, damaged samples removed, and the peas ground with a pestle and mortar in pH6.5 phosphate buffer (2ml/g) with purified, acid-washed sand. The resulting slurry was centrifuged for 10 minutes at 3000rpm, and the supernatant stored on ice.

(ii) Fresh peas were shelled and blended 3 times with cold acetone to remove all pigments. The pulp was filtered dry on sintered glass and a quantity of powder corresponding to 10g fresh peas extracted with 20ml pH6.5 phosphate buffer for 30 minutes, and centrifuged.

(iii) 12 pea seeds (variety Yates Earlicrop) were soaked overnight in distilled water (net weight 7g) and ground with pestle and mortar with sand in 14ml pH6.5 phosphate buffer, and centrifuged.

Chlorophyll bleaching was carried out in duplicate flasks containing 22ml pH6.5 phosphate buffer, 2ml crude chlorophyll in acetone, 0.5ml linoleic acid in ethanol, and 0.5ml enzyme extract. Incubation and sampling was as previously described. Results are presented in Table 4.83.

Table 4.83 Chlorophyll Bleaching Activity in Pea Extract

Pea Samples	Percent Chlorophyll Bleaching	
	2 mins.	4 mins.
Fresh Pea	73.5	86.4
Acetone Powder	0.0	0.0
Pea Seed	76.7	90.4

30. Effect of pH on Chlorophyll Bleaching by Pea Extract

Flasks containing 22ml buffer of pH4.5 to 9.0, 2ml crude chlorophyll in acetone, 0.5ml linoleic acid in ethanol and 0.5ml enzyme extract from fresh peas (2ml distilled water/g), were prepared and solutions incubated and examined as described previously. Results are presented in Table 4.84

and shown in Graph 37.

Table 4.84 Effect of pH on Chlorophyll Bleaching by Pea Extract

Buffer pH	Percent Chlorophyll Bleaching			
	No Linoleic Acid		With Linoleic Acid	
	2 mins.	4 mins.	2 mins.	4 mins.
4.50	2.9	4.7	6.0	14.3
5.01	3.7	5.6	38.8	59.9
5.57	3.7	5.7	57.6	70.3
5.99	5.8	7.9	74.9	87.1
6.53	4.0	5.7	79.1	9(.8
7.00	0.6	1.4	34.1	46.6
8.05	0.2	0.5	1.0	3.7
9.03	3.1	4.0	5.1	8.0

31. Effects of Incubation Time and Enzyme Level on Chlorophyll Bleaching and Peroxidation by Pea and Soya Extracts

31.1 Chlorophyll Bleaching

Flasks were prepared containing 2.0ml crude chlorophyll in acetone, 0.5ml linoleic acid in ethanol, volumes of pea extract (4ml pH6.5 buffer/g peas) from 0.0 to 2.0ml, and pH6.5 buffer to a total volume of 25.0ml. Aliquots were taken at incubation times from 0 to 10 minutes, and percent chlorophyll bleaching calculated. Results are presented in Table 4.85, and shown in Graph 38.

Table 4.85 Effect of Incubation Time and Enzyme Level on Chlorophyll Bleaching by Pea Extract

Volume of Pea Extract (ml)	Percent Chlorophyll Bleaching						
	1 min	2 min	3 min	4 min	5 min	7.5min	10min
0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.05	2.9	3.8	6.0	9.2	12.8	17.4	20.7
0.1	5.4	10.0	16.2	24.8	27.8	35.5	38.7
0.2	12.0	26.1	36.1	43.6	49.4	52.9	56.2
0.5	37.1	59.6	69.1	75.1	77.8	81.8	83.1
1.0	58.5	70.0	77.4	83.0	86.3	89.6	91.2
2.0	54.7	68.5	76.4	82.0	85.8	88.7	90.0

31.2 Peroxidation

31.21 Pea Extract

Aliquots (0.0 to 1.0ml) of pea extract (2ml pH6.5 buffer/g peas) were added to 0.5ml linoleic acid and pH6.5 buffer in a total volume of 25.0ml. After incubation for 0 to 10 minutes, 1ml aliquots were removed and added to 2ml absolute ethanol, the solutions made up to 10.0ml with 60% v/v ethanol, and centrifuged 10 minutes at 3000rpm. Absorbances were read at 234nm and 280nm on a Unicam S.P.800 against a blank of 60% ethanol. Control solutions containing buffer, acid and heat-inactivated enzyme solution were prepared, and their absorbances determined. Absorbances, corrected to give zero values at 350nm, are given in Table 4.86.

Table 4.86 Ultraviolet Absorption of Linoleic Acid Solutions Incubated with Pea Extract

Volume of Pea Extract (ml)	Absorbance at 234nm				Absorbance at 280nm			
	1 min	2 min	5 min	10 min	1 min	2 min	5 min	10min
0.05	0.060	0.125	0.270	0.420	0.000	0.016	0.052	0.080
0.1	0.185	0.314	0.538	0.714	0.035	0.070	0.154	0.208
0.2	0.420	0.570	0.828	1.020	0.095	0.175	0.310	0.370
0.5	0.510	0.590	0.980	1.180	0.160	0.238	0.438	0.505
1.0	0.525	0.615	0.915	1.135	0.195	0.250	0.450	0.530

31.22 Soya Extract

Aliquots (0.0 to 1.0ml) of soya extract were added to 0.5ml linoleic acid and pH6.5 buffer (23.5 to 24.5ml) and incubated 0 to 10 minutes. Sampling and analysis was as described above. Ultraviolet absorption at 234nm and 280nm were recorded after diluting 5 times with 60% ethanol.

Lipid peroxide was determined by the ferric thiocyanate method using 1ml 60% ethanol solution (before dilution) as previously described (Expt. 22.24). Results are presented in Table 4.87 and shown in Graph 39.

Table 4.87 Peroxide Formation with Linoleic Acid Incubated with Soya Extract

Method of Estimation	Volume of Soya Extract (ml)	Absorbance 1 min	2 min	5 min	10 min
UV Absorption at 234nm	0.1	0.140	0.240	0.400	0.380
	0.2	0.240	0.330	0.410	0.380
	0.5	0.500	0.550	0.480	0.490
	1.0	0.680	0.670	0.680	0.640
UV Absorption at 280nm	0.1	0.045	0.065	0.105	0.125
	0.2	0.095	0.090	0.170	0.165
	0.5	0.150	0.165	0.170	0.165
	1.0	0.225	0.215	0.215	0.220
Ferric Thiocyanate Absorption at 480nm	0.1	0.165	0.257	0.339	0.383
	0.2	0.210	0.293	0.315	0.294
	0.5	0.267	0.315	0.301	0.294
	1.0	0.251	0.232	0.249	0.225

32. Effect of Oxidised Linoleic Acid on Chlorophyll Bleaching and Peroxidation by Pea Extract

32.1 Preparation of Oxidised Linoleic Acid

One gram pure linoleic acid (Lot 10-P, Hormel Foundation) was dissolved in 10ml absolute ethanol, and 80ml 0.1M borate buffer (pH9.0) added. 20mg pure lipoxygenase preparation (Batch 22474, Koch-Light Laboratories, 80,000 units/mg) was added and the mixture incubated in a 150ml erhlenmeyer flask overnight with oxygen continually bubbled through the solution. The oxidised acid was made up to 100ml with pH9 buffer to give a solution containing 10mg/ml. One ml of this solution was diluted to 25ml with buffer, an aliquot (1ml) removed and added to 2ml absolute ethanol, and diluted to 10ml with 60% ethanol. The solution gave an absorbance of approximately 1.0 at 234nm after centrifugation. There was no prominent peak at 275-280nm. A small absorption plateau appeared at 275-280nm after standing for 3-4 hours.

32.2 Chlorophyll Bleaching

Chlorophyll bleaching was determined in solutions containing 5mg normal or oxidised linoleic acid and 0.5ml pea extract (2ml pH6.5 buffer/g peas). Results are presented in Table 4.88.

Table 4.88 Effect of Oxidised Linoleic Acid on Chlorophyll Bleaching by Pea Extract

Flask No.	Contents	Percent Chlorophyll Bleaching 2 mins.	4 mins
1	5mg linoleic acid + 0.5ml inactivated enzyme	0.0	0.0
2	5mg linoleic acid + 0.5ml enzyme	70.3	86.5
3	5mg oxidised linoleic acid + 0.5ml inactivated enzyme	0.0	0.0
4	5mg oxidised linoleic acid + 0.5ml enzyme	72.4	81.5

32.3 Peroxidation

Aliquots of pea extract (0.to 1.0ml) were added to 1.0ml oxidised linoleic acid in pH6.5 buffer (total volume 25ml) and incubated for up to 10 minutes. 1ml aliquots were diluted as described previously, and absorbances of the solutions measured at 234nm and 280nm against a blank of 60% ethanol. Results are presented in Table 4.89. Spectra of oxidised linoleic acid are shown in Graph 40.

Table 4.89 Peroxide Estimation in Oxidised Linoleic Acid Incubated with Pea Enzyme

Method of Estimation	Volume of Enzyme (ml)	Absorbance 0 min	1 min	2 min	5 min	10 min
UV absorption at 234nm	0.0	1.008	1.008	0.988	0.968	0.960
	0.05	1.008	1.098	1.090	1.140	1.158
	0.1	1.008	0.925	0.905	0.992	1.054
	0.2	1.008	0.970	1.048	1.125	1.158
	0.5	1.008	1.170	1.240	1.236	1.185
	1.0	1.008	1.312	1.378	1.312	1.268
UV absorption at 280nm	0.0	0.168	0.160	0.146	0.140	0.098
	0.05	0.168	0.198	0.148	0.218	0.190
	0.1	0.168	0.190	0.170	0.268	0.316
	0.2	0.168	0.194	0.266	0.350	0.380
	0.5	0.168	0.302	0.386	0.490	0.518
	1.0	0.168	0.414	0.522	0.574	0.566

33. Effect of Hemin and Oxidised Linoleic Acid on Chlorophyll Bleaching by Pea Extract

Flasks were prepared containing pH6.5 buffer, crude chlorophyll, 5mg normal or oxidised linoleic acid, 0.5ml acetone solution of hemin (crystalline, Koch-Light, 10^{-4}M), and 0.5ml pea enzyme (4ml pH6.5 buffer/g peas) so that each flask contained the same quantity of solvents (i.e. buffer,

acetone, ethanol). Chlorophyll bleaching was measured as described previously. Results are presented in Table 4.90.

Table 4.90 Effect of Hemin and Oxidised Linoleic Acid on Chlorophyll Bleaching by Pea Extract

Flask No.	Contents	Percent Chlorophyll Bleaching 1min	2min	4min	10min
1	5mg linoleic acid + 0.5ml inactivated enzyme	0.0	0.0	0.0	0.0
2	5mg linoleic acid + 0.5ml enzyme	25.2	49.6	69.3	79.3
3	5mg linoleic acid + 0.5ml hemin	0.0	0.0	0.0	0.0
4	5mg linoleic acid + 0.5ml hemin + 0.5ml enzyme	41.1	62.7	72.2	82.7
5	5mg oxidised linoleic acid + 0.5ml inactivated enzyme	0.0	0.0	0.0	0.0
6	5mg oxidised linoleic acid + 0.5ml enzyme	22.3	41.8	61.9	69.3
7	5mg oxidised linoleic acid + 0.5ml hemin	27.4	32.6	31.2	30.3
8	5mg oxidised linoleic acid + 0.5ml hemin + 0.5ml enzyme	44.8	59.5	69.5	71.5

34. Effect of Storage on Chlorophyll Bleaching and Lipoxygenase Activities in Frozen Vegetables

34.1 Chlorophyll Bleaching Activity

Samples of frozen peas and beans stored for periods up to 20 months at 15°F and -10°F, were examined for bleaching activity in the standard bleaching system used previously. Enzyme extracts were prepared from fresh and frozen samples (2ml pH6.5 buffer/g material). Results for stored unblanched peas (2 minutes incubation) are presented in Table 4.91 and shown in Graph 41. All other samples showed no chlorophyll bleaching activity.

34.2 Lipoxygenase Activity

Lipoxygenase activity was determined in 0.2ml enzyme extracts incubated with 24.3ml 0.1M phosphate buffer (pH8.0) and 0.5ml linoleic acid for 2 minutes. 1ml aliquots were diluted and absorbances measured at 234nm against a blank of 60% ethanol. Results of determinations for lipoxygenase activity expressed as absorbance at 234nm are presented in Table 4.91 and shown in Graph 41. All other samples showed no lipoxygenase activity.

Table 4.91 Chlorophyll Bleaching and Lipoxygenase Activities in Stored Frozen Unblanched Peas

Sample	Storage Temp. (°F)	Percent Chlorophyll Bleaching				
		Storage Time (Months)				
		0	3	8	13	20
Stored under N_2	15	69.8	46.3	26.3	7.7	1.8
Stored under O_2	"	69.8	39.0	20.4	7.1	0.7
Stored under N_2	-10	69.8	58.5	47.1	42.9	34.9
Stored under O_2	"	69.8	51.5	35.1	33.5	29.9

Sample	Storage Temp. (°F)	Absorbance at 234nm				
		Storage Time (Months)				
		0	3	8	13	20
Stored under N_2	15	0.610	0.585	0.460	0.290	0.125
Stored under O_2	"	0.610	0.530	0.400	0.215	0.060
Stored under N_2	-10	0.610	0.610	0.550	0.495	0.430
Stored under O_2	"	0.610	0.590	0.520	0.440	0.365

35. Chromatography of Acidified Pigment Extracts from Stored Frozen Peas

35.1 Unblanched Peas Stored 20 Months at 15°F

Pigments were extracted repeatedly with acetone from unblanched peas stored 20 months at 15°F under oxygen. The acetone extract was acidified with hydrochloric acid, evaporated to dryness under reduced pressure, and dissolved in a small volume of dry diethyl ether. The pigment extract was streaked on thin layers of cellulose and chromatographed for 15cm with solvent A. Separated pigment bands were rechromatographed on cellulose with solvent A or B, the pigments eluted with diethyl ether and their spectra recorded. Results are presented in Table 4.92.

Table 4.92 Pigments in Acidified Extract from Unblanched Peas Stored 20 Months at 15°F

Band	Colour	Distance Travelled (cm)	Absorption Maxima (nm)	Solvent	Identity
F1	pale yellow	14.5-15.0	424,450,478	n-hexane	β-carotene
F2	pale grey	13.5-13.75	410,667	diethyl ether	pheophytin a
F3	pale yellow	11.5-12.0	422,446,476	n-hexane	lutein
F4	pale yellow -green	11.0-11.25	434,653	diethyl ether	pheophytin b
F5	faint yellow	6.25- 6.5	insufficient material	-	unidentified
F6	grey	2.75- 4.0	409,466,506,534, 564,610,667	diethyl ether	pheophor-bide a
F7	yellow-green	1.5- 2.5	412,434,526,558, 600,654	diethyl ether	pheophor-bide b
F8	pale yellow -brown	0.0- 1.0	insufficient material	-	unidentified

35.2 Blanched Peas Stored 20 Months at -10°F

Pigments were extracted with acetone from blanched peas stored under nitrogen for 20 months at -10°F, the solution acidified and evaporated, the pigments dissolved in dry diethyl ether and chromatographed for 15cm on thin layers of cellulose with solvent A. The pigments were eluted from the adsorbent with diethyl ether and their spectra recorded. Results are presented in Table 4.93.

Table 4.93 Pigments in Acidified Extract from Blanched Peas Stored 20 Months at -10°F

Band	Colour	Distance Travelled (cm)	Absorption Maxima (nm)	Solvent	Identity
F9	yellow	14.5-15.0	424,450,478	n-hexane	β-carotene
F10	grey	12.5-13.5	410,468,505,532, 560,610,667	diethyl ether	pheophytin a
F11	yellow	11.75-12.25	422,446,476	n-hexane	lutein
F12	yellow-green	10.75-11.5	412,434,524,558, 600,653	diethyl ether	pheophytin b
F13	faint yellow	10.25-10.5	insufficient sample	-	violaxanthin?
F14	pale yellow	4.75- 5.0	insufficient sample	-	neoxanthin?

36. Chromatography of Pigments from Bleaching Systems

36.1 Pheophytin a

Pure pheophytin a was prepared as previously described by column and T.L.C. on sugar and cellulose respectively. Flasks were prepared containing 7.0ml pH6.5 phosphate buffer, 2ml acetone solution pheophytin a, 0.5ml linoleic acid in ethanol, and 0.5ml soya extract (5ml pH6.5 buffer/g soya bean meal), and incubated in the dark for 5, 30 and 120 minutes. Additional acid and enzyme (0.5ml) were added to one flask after 60

minutes incubation. Control solutions containing buffer, pigment, linoleic acid and heat-inactivated enzyme were incubated at the same time. After incubation, 30ml acetone was added to each flask, 10ml diethyl ether added, and the pigments phased into the ether layer by the addition of 10% sodium chloride. The ether solutions were thoroughly washed with sodium chloride solution and distilled water, and dried over anhydrous sodium sulphate. The pigment extracts were streaked on thin layers of cellulose and chromatographed for 15cm with solvent B. Separated pigment bands were rechromatographed on cellulose thin layers where sufficient material was available, and their absorption spectra recorded in diethyl ether. Results are presented in Table 4.94. Absorption spectra are given in Graphs 42 and 43.

Table 4.94 Pigments from Pheophytin a Incubated with Linoleic Acid and Soya Extract

Incubation Time (minutes)	Band	Colour	Distance Travelled (cm) (Solvent B)	Absorption Maxima in Diethyl Ether (nm)
0	BL1	grey	14.0-15.0	410,470,506,534,562, 609,667
5	BL2	grey	14.0-15.0	410,468,507,534,562, 610,667
	BL3	pale yellow-brown	11.5-12.0	408,504,533,658
	BL4	pale yellow-brown	10.5-11.0	408,504,533,658
	BL5	pale yellow	9.0- 9.5	408,504,533,658
	BL6	pale yellow	4.5- 5.0	-
	BL7	faint yellow-brown	0.5- 1.0	-
30	BL8	grey	14.0-15.0	409,467,507,534,559, 609,666
	BL9	yellow-brown	2.5- 5.5	664
	BL10	pale yellow-brown	0.5- 1.5	664
120	BL11	pale grey	14.5-15.0	409,468,506,534,562, 608,666
	BL12	yellow-brown	0.5- 2.0	-

36.2 Pheophytin b

Purified pheophytin b was incubated with linoleic acid and soya extract for 5, 30 and 120 minutes as described for pheophytin a. The bleached pigments were transferred to diethyl ether, and chromatographed on thin-layers of cellulose for 15cm with solvent A. The separated pigment bands were rechromatographed and absorption spectra determined in diethyl ether. Results are presented in Table 4.95. Spectra are shown in Graphs 44 and 45.

Table 4.95 Pigments from Pheophytin b Incubated with Linoleic Acid and Soya Extract

Incubation Time (minutes)	Band	Colour	Distance Travelled (cm) (Solvent A)	Absorption Maxima in Diethyl Ether (nm)
0	BL13	yellow-green	11.0-12.0	412,434,526,557,600,653
5	BL14	yellow-green	11.25-12.0	412,434,524,557,600,653
	BL15	pale yellow	6.5 - 7.0	428,655
	BL16	pale yellow	5.25- 5.5	427,655
30	BL17	yellow-green	11.25-12.0	412,434,526,558,600,653
	BL18	yellow-green	6.0 - 7.0	(408),428,519,553,600,655
	BL19	yellow-green	5.0 - 5.5	(408),427,518,552,601,655
	BL20	pale yellow	3.5 - 4.0	427,517,550,600,655
	BL21	pale yellow	2.5 - 2.75	426,516,600,655
	BL22	pale brown	0.5 - 1.0	428,517,(552),598,652
120	BL23	yellow-green	11.0 -11.50	412,534,526,557,600,653
	BL24	yellow-green	6.0 - 7.0	(408),428,520,553,600,655
	BL25	yellow-green	5.25- 5.75	427,519,600,654
	BL26	yellow-brown	0.5 - 1.5	431,(520),598,650

5. BIBLIOGRAPHY

ADAMS, H.W. and YAWGER, E.S. (1961)
Food Technol. 15, 314

ALLEN, M.B. (1966)
"The Chlorophylls", Vernon, L.P. & Seely, G.R. Eds.
Ch.16, p511, Academic Press Inc. N.Y.

AMES, G.R. and KING, A.T. (1966)
J. Sci. Food. Agr. 17, 301

ANDERSON, J.M. and BOARDMAN, N.K. (1966)
Biochim. Biophys. Acta. 112, 403

ANDERSON, A.F.H. and CALVIN, M. (1962)
Nature, 194, 285

A.O.A.C. (1960)
"Official Methods of Analysis" 9th Ed.
Assoc. Offic. Agr. Chemists, Washington D.C.

ARDAO, C. and VENNESLAND, B. (1960)
Plant Physiol. 35, 368

ARNON, D.I. (1949)
Plant Physiol. 24, 1

ARONOFF, S. (1950a)
Botan. Rev. 16, 525

ARONOFF, S. (1950b)
Chem. Revs. 47, 175

ARONOFF, S. (1953)
Adv. in Food Res. 4, 133

ARONOFF, S. (1960)
"Encyclopedia of Plant Physiology", Ruhland, W. Ed,
5 (1), 234, Springer, Berlin

ARONOFF, S. (1962)
Biochim. Biophys. Acta. 60, 193

ARONOFF, S. (1966)
"The Chlorophylls", Vernon, L.P. and Seely, G.R., Eds.,
Ch.1, p3, Academic Press Inc. N.Y.

BACON, M.F. (1964)
Rep. Rothamsted Exp. Stn. for 1963, 97

BACON, M.F. (1965a)
J. Chromatog. 17, 322

BACON, M.F. (1965b)
Rep. Rothamsted. Exp. Stn. for 1964, 118

BACON, M.F. (1966)
Biochem. J. 101, 34c

BACON, M.F. (1967)
Rep. Rothamsted. Exp. Stn. for 1966, 103

BACON, M.F. (1968)
Rothamsted. Exp. Stn. Personal Communication

BACON, M.F. and HOLDEN, M. (1966)
Rep. Rothamsted. Exp. Stn. for 1965, 108

BACON, M.F. and HOLDEN, M. (1967a)
Phytochem. 6, 193

BACON, M.F. and HOLDEN, M. (1967b)
Rep. Rothamsted Exp. Stn. for 1966, 102

BALL, C.O. and OLSON, F.C.W. (1957)
"Sterilisation in Food Technology"
1st Edn., McGraw Hill Book Co. Inc., N.Y.

BIGELOW, W.D., BOHART, G.S., RICHARDSON, A.C. and BALL, C.O. (1920)
National Canners Assoc. Bull. 16-L.

BILLMEYER, F.W. and SALTZMAN, M. (1966)
"Principles of Colour Technology", Interscience Publishers, N.Y.

BLAIN, J.A. and BARR, T. (1961)
Nature 190, 538

BLAIN, J.A. and STYLES, E.C.C. (1959)
Nature 184, 1141

BLAIN, J.A., HAWTHORN, J. and TODD, J.P. (1953)
J. Sci. Food Agric. 4, 580

BLAIR, J.S. (1940)
U.S. Patents 2,186,003; 2,189,774

BLIGH, E.G. and DYER, W.J. (1959)
Canad. J. Biochem. Physiol. 37, 911

BOARDMAN, N.K. and HIGHKIN, H.R. (1966)
Biochim. Biophys. Acta. 126, 189

BOGGS, M.M. and TALBURT, W.F. (1952).
Food Technol. 6, 438

BOGGS, M.M., DIETRICH, W.C., NUTTING, M.D., OLSON, R.L., LINDQUIST, F.E., BOHART, G.S., NEUMANN, H.J. and MORRIS, H.I. (1960)
Food Technol. 14, 181

BOGORAD, L. (1965)
"Chemistry and Biochemistry of Plant Pigments"
Goodwin, T.W., Ed., Ch.2, p29, Academic Press Inc., N.Y.

BOGORAD, L. (1966)
"The Chlorophylls", Vernon, L.P. and Seely, G.R., Eds.,
Ch.15, p481, Academic Press Inc., N.Y.

BORODIN, J. (1882)
Bot. Z. 40, 608

BOUMA, P.J. (1947)
"Physical Aspects of Colour", Elsevier Press, N.Y.

BOWMAN, F. and REMMENGA, E.E. (1965)
Food Technol. 19, 617

BRAVERMAN, J.B.S. (1963)
"Introduction to the Biochemistry of Foods", Ch.2, p6,
Elsevier Publ. Co., London.

BRIAT, B., SCHOOLEY, D.A., RECORDS, R., BUNNENBERG, E. and DJERASSI, C. (1967)
J. Amer. Chem. Soc. 89, 6170

BRODY, A.L., BEDROSIAN, K. and BALL, C.O. (1960)
Food Technol. 14, 552

BROWN, J.S. (1963)
Photochem. Photobiol. 2, 159

BROWN, S.R. (1968)
J. Fish. Res. Board. Canada 25, 523

BRUINSMA, J. (1963)
Photochem. Photobiol. 2, 241

BURNHAM, R.W., HANES, R.M. and BARTLESON, C.J. (1963)
"Colour: A Guide to Basic Facts and Concepts"
John Wiley and Sons, Inc., N.Y.

BURRELL, J.W.K., JACKMAN, L.M. and WEEDON, B.C.L. (1959)
Proc. Chem. Soc., 263

BURRELL, J.W.K., GARWOOD, R.F., JACKMAN, L.M., OSKAY, E., and WEEDON, B.C.L. (1966)
J. Chem. Soc., 2144

CHO, D.H. (1966)
Ph.D. Thesis, University of California, Davis, U.S.A.
(from Diss. Abstr. 27, 716B)

CLAYTON, R.K. (1966)
"The Chlorophylls", Vernon, L.P. and Seely, G.R., Eds.,
Ch.19, p609, Academic Press Inc., N.Y.

CLYDESDALE, F.M. (1966)
Ph.D. Thesis, University of Massachusetts, Amherst, U.S.A.

COLMAN, B. and VISHNIAC, W. (1964)
Biochim. Biophys. Acta. 82, 616

COMAR, C.L. and ZSCHEILE, F.P. (1942)
Plant Physiol. 17, 198

COMMISSION INTERNATIONALE de l'ECLAIRAGE (1931)
Proc. 8th Session, Cambridge, p19.

COMMITTEE ON COLORIMETRY, OPTICAL SOCIETY OF AMERICA (1944)
J. Opt. Soc. Amer. 34, 245

COMMITTEE ON COLORIMETRY, OPTICAL SOCIETY OF AMERICA (1953)
"The Science of Colour," Thomas Y. Crowell Co., N.Y.

CONANT, J.B., DIETZ, E.M., BAILEY, C.F. and KAMERLING, S.E. (1931)
J. Amer. Chem. Soc. 53, 2383

CORWIN, A.H. (1943)
"Organic Chemistry - An Advanced Treatise", Gilman, H., Ed.,
Ch.16, p1259, John Wiley & Sons Inc. N.Y.

CRABBE, P., DJERASSI, C., EISENBRAUN, E.J. and LIU, S. (1959)
Proc. Chem. Soc., 264

DAHLE, L.K., HILL, E.G. and HOLMAN, R.T. (1962)
Arch. Biochem. Biophys. 98, 253

DAVIDSON, J. (1954)
J. Sci. Food Agr. 5, 1

DECLAIRE, M. (1966)
Rev. Ferment. Ind. Aliment. 21, 95

DEROCHE, M.E. and COSTES, C. (1966)
Ann. Physiol. Veg. 8, 223

DEWEESE, D.G. (1962)
Ph.D. Thesis, Ohio State University, Ohio, U.S.A.
(From Diss. Abstr. 24,691B)

DIEHL, H.C. and BERRY, J.A. (1933)
Proc. Amer. Soc. Hort. Sci. 30, 496

DIETRICH, W.C. (1958)
Food Technol. 12, 428

DIETRICH, W.C. and NEUMANN, H.J. (1965)
Food Technol. 19, 1174

DIETRICH, W.D., LINDQUIST, F.E., BOHART, G.S., MORRIS, H.J. and NUTTING, M.D. (1955)
Food Res. 20, 480

DIETRICH, W.C., LINDQUIST, F.E. and BOGGS, M.M. (1957a)
Food Technol. 11, 485

DIETRICH, W.C., LINDQUIST, F.E., MIERS, J.C., BOHART, G.S., NEUMANN, H.J. and TALBURT, W.F. (1957b)
Food Technol. 11, 109

DIETRICH, W.C., NUTTING, M.D., OLSON, R.F., LINDQUIST, F.E., BOGGS, M.M. BOHART, G.S., NEUMANN, H.J. and MORRIS, H.J. (1959a)
Food Technol. 13, 136

DIETRICH, W.C., OLSON, R.L., NUTTING, M.D., NEUMANN, H.J. and BOGGS, M.M. (1959b)
Food Technol. 13, 258

DIETRICH, W.C., BOGGS, M.M., NUTTING, M.D. and WEINSTEIN, N.E. (1960)
Food Technol. 14, 522

DILLARD, M.G., HENICK, A.S. and KOCH, R.B. (1960)
Food Res. 25, 544

DILLARD, M.G., HENICK, A.S. and KOCH, R.B. (1961)
J. Biol. Chem. 236, 37

DOUGHERTY, R.C., STRAIN, H.H., SVEC, W.A., UPHAUS, R.A. and KATZ, J.J.
J. Amer. Chem. Soc. 88, 5037

EASTMOND, E.J., PETERSON, J.E. and STUMPF, R.R. (1951)
Food Technol. 5, 121

EDWARDS, R.A. (1963)
Ph.D. Thesis, Uni. of N.S.W.

EGLE, K. (1960)
"Encyclopedia of Plant Physiology", Ruhland, W. Ed., 5 (1), 323, 354,387, Springer, Berlin

EHEART, M.S. (1967)
J. Amer. Diet. Ass. 50, 207

EHEART, M.S. and GOTT, C. (1965)
Food Technol. 19, 867

ELAHI, M. (1967)
Ph.D. Thesis, Uni. of N.S.W.

EPSTEIN, A.I. (1959)
Ph.D. Thesis, Rutgers University, New Brunswick, U.S.A.

ERIKSSON, C.E. (1967)
J. Food. Sci. 32, 438

ESAU, P. (1958)
Food Technol. 12, 167

EVANS, R.M. (1948)
"An Introduction to Colour", John Wiley & Sons, Inc., N.Y.

FARKAS, D.F., GOLDBLITH, S.A. and PROCTOR, B.E. (1956)
Food Eng. 28, 52

FICKEN, G.E., JOHNS, R.B. and LINSTEAD, R.P. (1956)
J. Chem. Soc. 2272

FISCHER, H. and GOEBEL, S.C. (1936)
Ann. Chem. 524, 269

FISCHER, H. and HALBIG, P. (1926)
Ann. Chem. 448, 193

FISCHER, H. and KELLERMANN, H. (1935)
Ann. Chem. 519, 209

FISCHER, H. and LAMBRECHT, R. (1938)
Hoppe - Seyl. Z 253, 253

FISCHER, H. and ORTH, H. (1937)
"Die Chemie des Pyrroles" Vol.2, Pt.1, Akad. Verlagsges, Liepzig.

FISCHER, H. and PFEIFFER, H. (1944)
Ann. Chem. 555, 94

FISCHER, H. and STERN, A. (1940)
"Die Chemie des Pyrrols" Vol.2, Pt.2, Akad. Verlagsges, Liepzig.

FISCHER, H. and WENDEROTH, H. (1940)
Ann. Chem. 545, 140

FLEMING, I. (1967)
Nature 216, 151

FORMAN, L. and POKORNY, J. (1966)
Nahrung. 10, 267

FRENCH, C.S. (1958)
"Photobiology", Newburgh, R.W., Ed., p52, Oregon State College Press.

FRENCH, C.S. (1960)
"Encyclopedia of Plant Physiology", Ruhland, W., Ed., 5 (1), 252, Springer, Berlin

GAGE, R.S. and ARONOFF, S. (1956)
Plant Physiol. 31, 477

GILPIN, G.L., SWEENEY, J.P., CHAPMAN, V.J. and EISEN, J.N. (1959)
J. Amer. Diet. Assoc. 35, 359

GINI, B. and KOCH, R.B. (1961)
J. Food Sci. 26, 359

GOEDHEER, J.C. (1966)
"The Chlorophylls", Vernon, L.P. and Seely, G.R., Eds., Ch.13, p399, Academic Press Inc., N.Y.

GOLD, H.J. and WECKEL, K.G. (1959)
Food Technol. 13, 281

GOLDEN, J.H., LINSTEAD, R.P. and WHITHAM, G.H. (1958)
J. Chem. Soc. 1725

GOODWIN, T.W. (1955)
"Modern Methods of Plant Analysis", Paech, K. and Tracey, M.V., Eds., 3, 272, Springer, Berlin.

GOODWIN, T.W. (1965)
"Chemistry and Biochemistry of Plant Pigments", Academic Press Inc., N.Y.

GRANICK, S. (1965)
"Evolving Genes, Proteins", Symposium Rutgers State Uni., 1964, p67.

GROB, E.C. and SEILER, J. (1967)
Chimia 21, 466

GROSCH, W. (1967)
Z.Lebens - Untersuch. Forsch 135, 75

GUPTE, S.M. (1963)
Ph.D. Thesis, Uni. of Massachusetts, Amherst, U.S.A.

GUPTE, S.M. and FRANCIS, F.J. (1964)
Food Technol. 18, 1645

GUSS, P.L., RICHARDSON, T. and STAHMANN, M.A. (1968)
J. Amer. Oil. Chem. Soc. 45, 272

GUYER, R.B. and HOLMQUIST, J.W. (1954)
Food Technol. 8, 547

HAGER, A. (1957)
Planta 48, 592

HALL, J.L. and MACKINTOSH, D.L. (1964)
J. Food Sci. 29, 420

HARDY, A.C. (1936)
"Handbook of Colorimetry", M.I.T., Cambridge, Mass.

HARRIS, D.G. and ZSCHEILE, F.P. (1943)
Bot. Gaz. 104, 515

HAXO, F.T. and FORK, D.C. (1959)
Nature 184, 1047

HILL, R. (1963)
"Comprehensive Biochemistry", Florkin, M. and Stotz, E.H., Eds., 2, 73, Elsevier Publ. Co.

HILLS, G.L. and THIEL, C.C. (1946)
J. Dairy Res. 14, 340

HOLDEN, M. (1961)
Biochem. J. 78, 359

HOLDEN, M. (1962)
Biochim. Biophys. Acta. 56, 378

HOLDEN, M. (1963)
Photochem. Photobiol. 2, 175

HOLDEN, M. (1965a)
J. Sci, Food Agr. 16, 312

HOLDEN, M. (1965b)
"Chemistry and Biochemistry of Plant Pigments", T.W. Goodwin, Ed., Ch.17, p461, Academic Press Inc., N.Y.

HOLDEN, M. (1965c)
Rep. Rothamsted. Exp. Stn. for 1964, 117

HOLDEN, M. (1967a,b)
Rep. Rothamsted, Exp. Stn. for 1966, 101, 310

HOLLOWAY, G.L. (1966)
Aust. J. Dairy Tech. 21, 74

HOLT, A.S. (1958)
Canad. J. Biochem. Physiol. 36, 439

HOLT, A.S. (1965)
"Chemistry and Biochemistry of Plant Pigments", Goodwin, T.W. Ed., Ch.1, p3, Academic Press Inc., N.Y.

HOLT, A.S. (1966)
"The Chlorophylls", Vernon, L.P. and Seely, G.R., Eds., Ch.4, p111, Academic Press Inc., N.Y.

HOLT, A.S. and JACOBS, E.E. (1954)
Amer. J. Bot. 41, 710

HOLT, A.S. and MORLEY, H.V. (1959)
Canad. J. Chem. 37, 507

HUNTER, R.S. (1942)
National Bur. Stnds. Circ. C.429 (July 30)

HUNTER, R.S. (1958)
J. Opt. Soc. Amer. 48, 985

INADA, K. (1963)
Proceed. Crop. Sci. Soc. Japan, 32, 157

INADA, K. (1965)
Proceed. Crop. Sci. Soc. Japan, 33, 301

JEFFREY, S.W. (1961)
Biochem. J. 80, 336

JEFFREY, S.W. (1962)
Nature 194, 600

JEFFREY, S.W. (1963)
Biochem. J. 86, 313

JEFFREY, S.W. (1965)
C.S.I.R.O. Division of Fisheries and Oceanography.
Personal communication.

JENSEN, A. AASMUNDRUD, O. and EIMHJELLEN, K.E. (1964)
Biochim. Biophys. Acta. 88, 466

JOFFE, F.M., STIER, E.F., BONGOLAN, D.C., EPSTEIN, A.I. and BALL, C.O. (1961)
Food Technol. 15, 507

JONES, I.D., WHITE, R.C. and GIBBS, E. (1961)
Food Technol. 15, 172

JONES, I.D., WHITE, R.C. and GIBBS, E. (1962)
Food Technol. 16, 96 (3)

JONES, I.D., WHITE, R.C. and GIBBS, E. (1963)
J. Food Sci. 28, 437

JONES, I.D., WHITE, R.C., GIBBS, E.C. and DENARD, C.D. (1968)
J. Agr. Food Chem. 16, 80

JOSLYN, M.A. and MACKINNEY, G. (1938)
J. Amer. Chem. Soc. 60, 1132

JUDD, D.B. (1950)
National Bur. Stnds. Circ. 478

JUDD, D.B. and WYSZECKI, G. (1963)
"Colour in Business Science and Industry", 2nd. Ed.
John Wiley & Sons, Inc., N.Y.

KAHN, J.S. and BANNISTER, T.T. (1965)
Photochem. Photobiol. 4, 27

KAHN, J.S. and CHANG, I.C. (1965)
Photochem. Photobiol. 4, 733

KAPSALIS, J.G., DRIVER, M., JOHNSON, K.R., NU, I.T. and HENICK, A.S. (1965)
Food Technol. 19, 1149

KARRER, P. and JUCKER, E. (1950)
"Carotenoids". Transl. Braude, B.A., Elsevier Publ. Co., N.Y.

KATZ, J.J., DOUGHERTY, R.C. and BOUCHER, L.J. (1966)
"The Chlorophylls", Vernon, L.P. and Seely, G.R., Eds.,
Ch.7, p185, Academic Press Inc., N.Y.

KEFFORD, J.F. (1963)
C.S.I.R.O. Food Pres. Quart. 23, 8

KHAN, N.A. (1959)
J. Indian Chem. Soc. 36, 529

KHAN, N.A. (1965)
Oleagineux 20, 683

KIM, W.S. (1967)
Z. Naturforsch B. 22, 1054

KOCH, R.B., STERN, B. and FERRARI, C.G. (1958)
Arch. Biochem. Biophys. 78, 165

KOHN, H.I. and LIVERSEDGE, M. (1944)
J. Pharmacol. 82, 292

KOKATNUR, M.G., BERGAN, J.G. and DRAPER, H.H. (1966)
Proc. Soc. Exptl. Biol. Med. 123, 314

KOLESNIKOV, P.A. (1948)
Dokl. Akad. Nauk. S.S.S.R. 60, 1353

KOLESNIKOV, P.A. (1949)
Biokhimiya 14, 124

KRAMER, A. and TWIGG, B.A. (1962)
"Fundamentals of Quality Control for the Food Industry"
Ch.3, p19, AVI. Publ. Co.

KUPKE, D.W. and FRENCH, C.S. (1960)
"Encyclopedia of Plant Physiology", Ruhland, W., Ed.,
5(1), 298, Springer, Berlin

KWON, T.W. and OLCOTT, H.S. (1966)
J. Food Sci. 31, 552

KWON, T.W. and WATTS, B.M. (1963)
J. Food Sci. 28, 627

KWON, T.W. and WATTS, B.M. (1964)
J. Food Sci. 29, 294

KWON, T.W., MENZEL, D.B. and OLCOTT, H.S. (1965)
J. Food Sci. 30, 808

LAST, J.H. and SHIPTON, J. (1966)
C.S.I.R.O. Food Pres. Quart. 26, 44

LEA, C.H. (1952)
J. Sci. Food Agr. 3, 586

LEE, F.A. (1954)
Food Res. 19, 515

LEE, F.A. and MATTICK, L.R. (1961)
J. Food Sci. 26, 273

LEE, F.A. and WAGENKNECHT, A.C. (1951)
Food Res. 16, 239

LEE, F.A. and WAGENKNECHT, A.C., and HENING, J.C. (1955)
Food Res. 20, 289

LEE, F.A., WAGENKNECHT, A.C. and GRAHAM, R. (1956)
Food Res. 21, 666

LINDQUIST, F.E., DIETRICH, W.C. and BOGGS, M.M. (1950)
Food Technol. 4, 5

LITTLE, A.C., CHICHESTER, C.O. and MACKINNEY, G. (1958)
Food Technol. 12, 403

LIVINGSTON, B. (1957)
Food Technol. 11, 1

LIVINGSTON, B. (1959)
Food Eng. 31, 74, 98

LOEF, H.W. and THUNG, S.B. (1965)
Z. Lebens. -Untersuch. Forsch. 126, 401

LUH, B.S., LEONARDS, S., SIMONE, M. and VILLARREAL, F. (1964)
Food Technol. 18, 363

LUNDBERG, W.O. (1961)
"Autoxidation and Antioxidants" Vol.I
Interscience Publ. Inc., N.Y.

LWOWSKI, W. (1966)
"The Chlorophylls", Vernon, L.P. and Seely, G.R., Eds.,
Ch.5, p119, Academic Press Inc., N.Y.

LYNCH, L.J. and MITCHELL, R.S. (1953)
C.S.I.R.O. Bull. No.273

LYNN CO, D.Y.C., and SCHANDERL, S.H. (1967a)
Phytochem. 6, 145

LYNN CO, D.Y.C., and SCHANDERL, S.H. (1967b)
J. Chromatog. 26, 442

MACKINNEY, G. (1940)
J. Biol. Chem. 132, 91

MACKINNEY, G. (1941)
J. Biol. Chem. 140, 315

MACKINNEY, G. and CHICHESTER, C.O. (1954)
Adv. in Food Res. 5, 302

MACKINNEY, G. and JOSLYN, M.A. (1940)
J. Amer. Chem. Soc. 62, 231

MACKINNEY, G. and JOSLYN, M.A. (1941)
J. Amer. Chem. Soc. 63, 2530

MACKINNEY, G. and LITTLE, A.C. (1962)
"Colour of Foods", AVI Publ. Co. Conn.

MACKINNEY, G. and WEAST, C.A. (1940)
Ind. Eng. Chem. 32, 392

MALECKI, G.J. (1957)
Brit. Patent. 772, 062

MALECKI, G.J. (1964)
Food Eng. 36, 93

MANNING, W.M. and STRAIN, H.H. (1943)
J. Biol. Chem. 151, 1

MAPSON, L.W. and MOUSTAFA, E.M. (1955)
Biochem. J. 60, 71

MARKS, G.S. (1966)
Bot. Rev. 32, 56

MASURE, M.P. and CAMPBELL, H. (1944)
Fruit Prod. J. 23, 369

MAYER, H. (1930)
Planta 11, 294

McCONNELL, J.E.W. (1956)
National Canners Assoc. Bull. D-582

MICHEL-WOLWERTZ, M.R. and SIRONVAL, C. (1965)
Biochim. Biophys. Acta. 94, 330

MICHIGAN STATE UNIVERSITY (19--)
CORE program of the Computor Laboratories, Agricultural Experiment Station, Michigan State University, Lansing, U.S.A.

MOLISCH, H. (1896)
Ber. Deut. Botan. Ges. 14, 16

MONTEIRO, H.J. (1965)
J. Chromatog. 18, 594

MUNSELL BOOK OF COLOUR (1965)
Neighbouring Hues Edition, Munsell Colour Co. Inc., Md.

NATIONAL CANNERS ASSOCIATION (1954)
"A Laboratory Manual for the Canning Industry"
N.C.A. Res. Lab. Washington.

NATIONAL CANNERS ASSOCIATION (1955)
Bulletin 30-L (2nd Ed.) N.C.A. Res. Lab. Washington.

NICKERSON, D. (1940)
J. Opt. Soc. Amer. 30, 575

NICKERSON, D. (1946)
U.S. Dept. Agr. Misc. Publ. 580

NUTTING, M.D. and BECKER, R. (1966)
J. Food Sci. 31, 210

NUTTING, M.D., VOET, M. and BECKER, R. (1965)
Anal. Chem. 37, 445

O'BRIEN, P.J. and FRAZER, A.C. (1966)
Proc. Nutr. Soc. 25, 9

OGAWA, T. and SHIBATA, K. (1965)
Photochem. Photobiol. 4, 193

PARK, R.B. (1966)
"The Chlorophylls", Vernon, L.P. and Seely, G.R., Eds.,
Ch.9, p283, Academic Press Inc., N.Y.

PATTON, S., KEENEY, M. and KURTZ, G.W. (1951)
J. Amer. Oil. Chem. Soc. 28, 391

PENNINGTON, F.C., STRAIN, H.H., SVEC, W.A. and KATZ, J.J. (1964)
J. Amer. Chem. Soc. 86, 1418

PENNINGTON, F.C., STRAIN, H.H., SVEC, W.A. and KATZ, J.J. (1967)
J. Amer. Chem. Soc. 89, 3875

PERKINS, H.J. and ROBERTS, D.W.A. (1962)
Biochim. Biophys. Acta. 58, 486

PERKINS, H.J. and ROBERTS, D.W.A. (1964)
Biochim. Biophys. Acta. 79, 20

PETERSON, P.D. and McKINNEY, H.H. (1938)
Phytopath. 28, 329

PINSENT, B.R.W. (1962)
J. Food Sci. 27, 120

POHLE, W.D., GREGORY, R.L. and Van GIESSEN, B. (1964)
J. Amer. Oil. Chem. Soc. 41, 649

PRIVETT, O.S., NICKELL, C., LUNDBERG, W.O. and BOYER, P.D. (1955)
J. Amer. Oil. Chem. Soc. 32, 505

QUARTERMASTER FOOD AND CONTAINER INSTITUTE (1954)
"Colour in Foods" Symposium, Chicago, Illinois.

RABINOWITCH, E.I. (1945)
"Photosynthesis and Related Processes" Vol.I, Interscience Publ., Inc., N.Y.

RABINOWITCH, E.I. (1951)
"Photosynthesis and Related Processes" Vol.2, Pt.I, Interscience Publ., Inc., N.Y.

RABINOWITCH, E.I. (1956)
"Photosynthesis and Related Processes" Vol.2, Pt.2, Interscience Publ., Inc., N.Y.

RABINOWITCH, E.I. (1963)
Misc. Publ. 1145, National Res. Council, Washington.

RAPOPORT, H. and HAMLOW, H.P. (1961)
Biochem. Biophys. Res. Commun. 6, 134

REED, G. (1966)
"Enzymes in Food Processing" Ch.9, p192, Academic Press Inc., N.Y.

RESENDE, R. (1966)
Ph.D. Thesis, Uni. of Massachusetts, Amherst, U.S.A.
(Diss. Abstr. 28, 2740B)

RHEE, K.S. and WATTS, B.M. (1966a,b)
J. Food Sci. 31, 664,675

ROSS, E., PAULS, R.H. and HARD, M.M. (1959)
Food Technol. 13, 711

ROUBAL, W.T. and TAPPEL, A.L. (1966)
Arch. Biochem. Biophys. 113, 5,150

SAN PIETRO, A. and BLACK, C.C. (1965)
Ann. Rev. Plant Physiol. 16, 155

SASLAW, L.D., ANDERSON, H.J. and WARAVDEKAR, V.S. (1963)
Nature 200, 1098

SASLAW, L.D., CORWIN, L.M. and WARAVDEKAR, V.S. (1966)
Arch. Biochem. Biophys. 114, 61

SATO, K., MIZUNO, S. and HIRAYAMA, M. (1967)
J. Org. Chem. 32, 177

SCHANDERL, S.H. (1962)
Ph.D. Thesis, Uni. of California, Davis, U.S.A.

SCHANDERL, S.H. and LYNN CO, D.Y.C. (1966)
J. Food Sci. 31, 141

SCHANDERL, S.H., CHICHESTER, C.O. and MARSH, G.L. (1962)
J. Org. Chem. 27, 3865

SCHANDERL, S.H., MARSH, G.L. and CHICHESTER, C.O. (1965)
J. Food Sci. 30, 312, 317

SCOFIELD, R. (1943)
Natl. Paint, Varnish, Lacquer Assoc., Sci. Sec. Circ. 664

SEELY, G.R. (1966)
"The Chlorophylls" Vernon, L.P. and Seely, G.R., Eds.,
Ch.3, p67, Academic Press, Inc., N.Y.

SEELY, G.R. and JENSEN, R.G. (1965)
Spectrochim. Acta. 21, 1835

SESTAK, Z. (1958)
J. Chromatog. 1, 293

SESTAK, Z. (1965)
Chromatog. Rev. 1, 65

SESTAK, Z. (1966a)
Biol. Plant Acad. Sci. Bohem. 8, 97

SESTAK, Z. (1966b)
"Le Chloroplaste" C. Sironval, Ed., Masson, Paris.

SHIMIZU, S. and TAMAKI, E. (1963)
Arch. Biochem. Biophys. 102, 152

SIDDIQI, A.M. and TAPPEL, A.L. (1956)
Arch. Biochem. Biophys. 60, 91

SIDDIQI, A.M. and TAPPEL, A.L. (1957)
J. Amer. Oil Chem. Soc. 34, 529

SIDWELL, C.G., SALWIN, H. and MITCHELL, J.M. (1955)
J. Amer. Oil Chem. Soc. 32, 13

SIEGELE, J. (1955)
Ph.D. Thesis, Oregon State College, Oregon, U.S.A.
(Gold and Weckel 1959)

SINNHUBER, R.O. and YU, T.C. (1958)
Food Technol. 12, 9

SINNHUBER, R.O. and YU, T.C. and YU, T.C. (1958)
Food Res. 23, 626

SIRONVAL, C. (1954)
Physiol. Plantarum, 7, 523

SMITH, E.L. (1942)
Chron. Bot. 7, 148

SMITH, J.H.C. and BENITEZ, A. (1955)
"Modern Methods of Plant Analysis", Paech, K. and Tracey, M.V.,
Eds., 4, 142, Springer, Berlin.

SMITH, J.H.C. and FRENCH, C.S. (1963)
Ann. Rev. Plant. Physiol. 14, 181

STANIER, R.Y. and SMITH, J.H.C. (1960)
Biochim. Biophys. Acta. 41, 478

STEELE, C.C. (1943)
"Organic Chemistry - an Advanced Treatise", Gilman, H., Ed.,
2nd. Ed. 2, 1293, John Wiley & Sons, Inc., N.Y.

STRAIN, H.H. (1951)
"Manual of Phycology", Smith, G.M., Ed., p243,
Chronica. Botanica. Co.

STRAIN, H.H. (1954)
Agr. Food Chem. 2, 1222

STRAIN, H.H. (1958)
32nd Annual Priestby Lecture - Penn. State Uni., Uni. Park, Penn.

STRAIN, H.H. (1967)
"Chromatography", Heftmann, E., 2nd. Ed.,
Ch.23, p.661, Reinhold Publ. Corp.

STRAIN, H.H. and MANNING, W.M. (1942a)
J. Biol. Chem. 146, 275

STRAIN, H.H. and MANNING, W.M. (1942b)
J. Biol. Chem. 144, 625

STRAIN, H.H. and SVEC, W.A. (1966)
"The Chlorophylls", Vernon, L.P. and Seely, G.R. Eds.,
Ch.2, p21, Academic Press Inc., N.Y.

STRAIN, H.H., MANNING, W.M. and HARDIN, G. (1943)
J. Biol. Chem. 148, 655

STRAIN, H.H., THOMAS, M.R. and KATZ, J.J. (1963)
Biochim. Biophys. Acta. 75, 306

STRAIN, H.H., SHERMA, J., BENTON, F.L. and KATZ, J.J. (1965)
Biochim. Biophys. Acta. 109, 1, 16, 23

STRAIN, H.H., SHERMA, J. and GRANDOLFO, M. (1967)
Anal. Chem. 39, 926

STRAIN, H.H., SHERMA, J. and GRANDOLFO, M. (1968)
Anal. Biochem. 24, 54

STRELL, M., KALAGANOFF, A. and KOLLER, H. (1960)
Angew. Chem. 72, 169

SUD'INA, E.G. (1961)
Abstracts 5th Inter. Congr. Biochem. p454

SUD'INA, E.G. (1963)
Photochem. Photobiol. 2, 181

SURREY, K. (1964)
Plant Physiol. 39, 65

SWEENEY, J.P. and MARTIN, M.E. (1958)
Food Res. 23, 635

SWEENEY, J.P. and MARTIN, M.E. (1961)
Food Technol. 15, 263

SWEENEY, J.P., CHAPMAN, V.J., MARTIN, M.E. and DAWSON, H.E. (1961)
Food Technol. 15, 341

TAN, C.T. and FRANCIS, F.J. (1962)
J. Food Sci. 27, 232

TAPPEL, A.L. (1961)
"Autoxidation and Antioxidants", Lundberg, W.O., Ed.,
1, 325, Interscience Publ.

TAPPEL, A.L. (1962a)
"Methods in Enzymology", Colowick, S.P. and Kaplan, N.O., Eds.,
5, 539, Academic Press Inc., N.Y.

TAPPEL, A.L. (1962b)
"Symposium on Foods: Lipids and their Oxidation", Schultz, H.W.,
Day, E.A. and Sinnhuber, R.O., Eds., Ch.6, p122, AVI Publ. Co. Conn.

TARLADGIS, B.G., WATTS, B.M., YOUNATHAN, M.T. and DUGAN, L.R. (1960)
J. Amer. Oil Chem. Soc. 37, 44

TARLADGIS, B.G., PEARSON, A.M. and DUGAN, L.R. (1962)
J. Amer. Oil Chem. Soc. 39, 34

TARLADGIS, B.G., PEARSON, A.M. and DUGAN, L.R. (1964)
J. Sci. Food Agr. 15, 602

THOMAS, W.E. (1928)
U.S. Patent. 1,685,511

TOLBERT, N.E. and BURRIS, R.H. (1950)
J. Biol. Chem. 186, 791

TOOKEY, H.L., WILSON, R.G., LOHMAR, R.L. and DUTTON, H.J. (1958)
J. Biol. Chem. 230, 65

TSWETT, M. (1906)
Ber. deut. Botan. Ges. 24, 384

TURNER, E.W., PAYNTER, W.D., MONTIE, E.J., BESSERT, M.W., STRUCK, G.M.
and OLSON, F.C. (1954)
Food Technol. 8, 326

UNITED STATES DEPARTMENT OF AGRICULTURE (1953)
U.S. Stnds. for Grades of Canned Green Beans

VAN BUREN, J.P., MOYER, J.C. and ROBINSON, W.B. (1964)
Food Technol. 18, 1204

VERNON, L.P. (1960)
Anal. Chem. 32, 1144

VERNON, L.P. and AVRON, M. (1965)
Ann. Rev. Biochem. 34, 269

VERNON, L.P. and SEELY, G.R. (1966)
"The Chlorophylls: Physical, Chemical and Biological Properties", Academic Press Inc., N.Y.

VOGEL, A.I. (1961)
"Practical Organic Chemistry", Longmans, Green & Co. Ltd. London.

WAGENKNECHT, A.C. (1957)
J. Amer. Oil. Chem. Soc. 34, 509

WAGENKNECHT, A.C. (1963)
J. Food Sci. 28, 489

WAGENKNECHT, A.C. and LEE, F.A. (1956)
Food Res. 21, 605

WAGENKNECHT, A.C. and LEE, F.A. (1958)
Food Res. 23, 25

WAGENKNECHT, A.C., LEE, F.A. and BOYLE, F.P. (1952)
Food Res. 17, 343

WALKER, G.C. (1964a,b)
J. Food Sci. 29, 383, 389

WASSINK, E.C. (1963)
"Comparative Biochemistry", Florkin, M. and Mason, H.W., Eds., 5, 347, Academic Press Inc., N.Y.

WEAST, C.A. and MACKINNEY, G. (1940)
J. Biol. Chem.133, 551

WELLER, A. (1954)
J. Amer. Chem. Soc. 76, 5819

WESTCOTT, D.E., LIVINGSTON, G.E., ESSELEN, W.B. and FELLERS, C.R. (1955)
Food Res. 20, 149

WHITE, R.C., JONES, I.D. and GIBBS, E. (1963)
J. Food Sci. 28, 431

WHITFIELD, F.B. and SHIPTON, J. (1966)
J. Food Sci. 31, 328

WHITTINGHAM, C.P. (1965)
"Chemistry and Biochemistry of Plant Pigments", Goodwin, T.W., Ed., Ch13, p357, Academic Press Inc., N.Y.

WICKLIFF, J.L. and ARONOFF, S. (1962)
Plant Physiol. 37, 584

WILLSTATTER, R. and HOCHEDER, F. (1907)
Ann. Chem. 354, 205

WILLSTATTER, R. and MIEG, W. (1906)
Ann. Chem. 350, 1

WILLSTATTER, R. and SJOBERG, K. (1924)
Z. Physiol. Chem. 138, 171

WILLSTATTER, R. and STOLL, A. (1910)
Ann. Chem. 378, 18

WILLSTATTER, R. and STOLL, A. (1911)
Ann. Chem. 387, 317

WILLSTATTER, R. and STOLL, A. (1913)
"Untersuchungen uber chlorophyll", Springer, Berlin.

WILLSTATTER, R. and STOLL, A. (1918)
"Assimilation der Kohlensaure," Springer, Berlin (from Schanderl 1962)

WILSON, J.R. and NUTTING, M.D. (1963)
Anal. Chem. 35, 144

WINTERSTEIN, A. and STEIN, G. (1933)
Hoppe-Seyl. Z. 220, 263

WOLF, H., BROCKMANN, H., BIERE, H. and INHOFFEN, H.H. (1967)
Ann. Chem. 704, 208

WOLKEN, J.J. (1963)
Misc. Publ. 1145, National Res. Council, Washington, p575

WOODWARD, R.B. (1960)
Angew. Chem. 72, 651

WOODWARD, R.B. (1961)
Pure Appl. Chem. 2, 383

WOODWARD, R.B. et al. (1960)
J. Amer. Chem. Soc. 82, 3800

WRIGHT, W.D. (1964)
"The Measurement of Colour", 3rd. Ed., Hilger & Watts Ltd., London

YU, R.T. (1966)
Ph.D. Thesis, Polytech. Inst. of Brooklyn, N.Y.
(Diss. Abstr. 26, 3651B)

YU, T.C. and SINNHUBER, R.O. (1964)
J. Amer. Oil Chem. Soc. 41, 540

YU, T.C. and SINNHUBER, R.O. (1967)
J. Amer. Oil Chem. Soc. 44, 256

ZIEGLER, R. and EGLE, K. (1965)
Beitr. Biol. Pflanz. 41, 11

ZSCHEILE, F.P. (1934a)
Bot. Gaz. 95, 529

ZSCHEILE, F.P. (1934b)
J. Phys. Chem. 38, 95

ZSCHEILE, F.P. and COMAR, C.L. (1941)
Botan. Gaz. 102, 463