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P. T. O.
Preamble

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3. These Regulations are subject to requirements of any body under whose sponsorship the research project giving rise to the thesis/dissertation is carried on.
AN ELECTRON MICROSCOPY STUDY
OF THE PARTICULATE MATERIAL IN
CITRUS FRUITS AND BEVERAGES.

by

GERRARD GRANVILLE JEWELL M.I.Biol.

Thesis submitted in partial fulfilment
of the requirements for the degree of
Ph.D. of the University of Surrey.

Research conducted at
The Food Research Association,
Leatherhead, Surrey.

Submitted: June 1975.
SUMMARY

The structure of fresh orange tissue, juices, comminuted bases and products has been studied by microscopy. The techniques employed were light microscopy, thin sectioning, negative staining, freeze etching and scanning electron microscopy. Physical and chemical parameters of the particles in the juices, bases and products were also measured and these included cloud level, estimation of hesperidin content and refractive index of hesperidin.

The appearance of the cells found in the flavedo, albedo, segment membrane and juice sac were sufficiently characteristic to enable the origin of the various cellular fragments found in the processed juice to be ascribed to a particular tissue origin. The structures observed in the oil ring formed in the neck of bottles of certain beverages was found to be mainly lipid materials. The form of the lipid was different on beverages with contrasting processing history. Numerous aggregates of oil droplets and hesperidin crystals were observed in the cloudy portion of the beverages, and a substantial number of these particles were less than 0.5 μm diameter.

A method was evolved for estimating the crystalline hesperidin content of the sample based on the difference in the ultraviolet absorption of a methanol and aqueous extract of the beverage.

It was concluded that the quantity of hesperidin present in orange beverages is an important, although not the sole factor in determining its cloud level. Samples with a high cloud level contained high levels of hesperidin.
A limited study of lemon beverages indicated that hesperidin content may not play such an important role in their cloud level.
ACKNOWLEDGEMENTS

The work contained in this thesis was undertaken as a collaborative industrial study, and it is a pleasure to be able to record my sincere thanks to my numerous colleagues who have helped me in many ways.

Firstly, to Professor A.W. Holmes, Director of the Food Research Association, for permission to submit the work, and to Professor J. Smith of the Department of Microbiology for making available the facilities for the collaborative study.

Secondly, to Professor A.W. Holmes and Dr. M. Butler for acting as my supervisors, and their helpful comments and discussions during the course of the work.

Thirdly, to Miss Jane Heathcock and Miss Clare Pettman who provided skilled technical assistance during various stages of the microscopy work. To Mr. A.R. Hines for his patient preparation of the photographs and to Mrs. Anne Burrows for her careful typing of the thesis.

Fourthly, to the following companies who provided the samples studied during the project; Beechams Ltd. (Mr. Robbins), A.E. Bevan Ltd. (Mr. Goddard), Coca Cola Co. (Mr. Sherry) and J. Lyons and Co. (Mr. Lampitt).

Finally, to my wife for her constant encouragement and understanding during this project and my previous part time studying.
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INTRODUCTION

Historical and Technological Aspects

The various species of the genus Citrus are all believed to be native to the subtropical and tropical regions of Asia and the Malay Archipelago, and to have spread from there to other parts of the world.

The first member of the Citrus group to be documented in European civilisation was the Citron (Citrus Medica L), mentioned in about 300 B.C., by Theophrastus (Webber, 1943) and it is from this authoritative review that much of the subsequent historical comment is drawn. For hundreds of years this appears to have been the only citrus fruit known in Europe. Then came in the following order the sour orange (Citrus Aurantium L) in about 900 A.D., the lemon (Citrus Lemon L) at about the same period, and the sweet orange (Citrus Sinensis L) not until approximately 1400 A.D. Despite the comparatively late arrival of the sweet orange in Europe, it is apparently clear from ancient Chinese literature that those parts of the world where the fruit was indigenous, recognised the benefits to be obtained from cultivating the fruit, and mention is made of the harvest of oranges in an old Chinese book, the "Yu Kung", dated about 2200 B.C.

The citrus fruits became rapidly established in Mediterranean Europe during the fifteenth century, and in 1493 were introduced into America by Columbus. The current importance of citrus products may be assessed from some recent statistics (Commonwealth Secretariat 1972) which show that the annual production of all types of citrus fruit exceeds 30 million tons, of which about one quarter is processed to
give some 700 to 750 million gallons of juice. The value of the juices is around £250 million and as such accounts for rather more than half the total world business in fruits of all kinds.

The development of a highly sophisticated citrus industry began when it was recognised that greater yields of fruit could be obtained by growing sweet oranges on either sour orange or rough lemon root stock. The introduction of new root stocks not only increased the yield per tree but also the range of suitable geographical locations for citrus production. Thus we now have citrus grown for major commercial use in Africa, Australia, the Americas (Argentina, Brazil and North America), Cyprus, China, Greece, Egypt, French Morocco, Italy, Israel, Japan, Sicily, Spain and Tunisia.

As mentioned above, an important use for citrus fruits is in the production of juices to be consumed as beverages, this being particularly true of the sweet orange. Indeed, several major technical innovations were required in order to be able to produce an acceptable juice on a commercial scale. If juice is extracted by hand from a fresh orange, one obtains a watery, yellow, somewhat cloudy liquid whose soluble sugars give a sucrose equivalent of 11° Brix (Sinclair, 1961). The juice will contain a very broad spectrum of constituent compounds, as might be expected from a product which has been obtained by the degradation of a living fruit tissue. These compounds will include sugars, proteins, lipids including essential oils, organic acids, colouring matters, vitamins, flavanoids, minerals and a considerable amount of pectinaceous material. This complex solution is to most people a refreshing beverage with a highly characteristic flavour.
As was mentioned above, the juice is obtained by the degradation of the fruit tissues, so it is pertinent to consider the structural organisation of the orange. A brief description of the various anatomical elements and their relationship to each other will be presented here, whilst a more detailed consideration of the cell types found in these structures is presented in a later part of this introduction. The various tissues of the orange are depicted schematically in Fig 1. The outermost orange coloured tissue is called the flavedo. It is just below the surface of the flavedo that the oil glands which contain the bulk of the essential oil found in the orange are located. The next layer of the orange peel which is located immediately below the flavedo is the thick white layer called the albedo. Contained within this peel are the segments or carpels which contain the juice sacs and seeds. Each segment is surrounded by a relatively tough segment membrane. The final tissue which is recognised in the fresh orange is the central core. When juice is extracted from the orange, some of the tissues which are ruptured remain adhering together and are described in technological terms as the rag and pulp. These elements are recognised as consisting of the following tissues from the fresh fruit. The rag is the central column and segment membranes, whilst the pulp is the empty juice sacs. The relative proportion of these tissues is given in Table I which is adapted from the results of Sinclair (1961) and Kefford and Chandler (1970).

<table>
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<th>Tissue</th>
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<tr>
<td>Flavedo</td>
<td>10%</td>
</tr>
<tr>
<td>Albedo</td>
<td>12-30%</td>
</tr>
<tr>
<td>Edible Portion</td>
<td>50-80%</td>
</tr>
<tr>
<td>(a) Juice</td>
<td>35-55%</td>
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<tr>
<td>(b) Rag</td>
<td>12%</td>
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<tr>
<td>(c) Pulp</td>
<td>8-12%</td>
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Fig. 1. Schematic view of a cross section of an orange.
The industry and technology which has developed for the production of orange juice will now be briefly considered. The European fruits are of basically two distinct cultivars – Valencias (traditionally from Spain and Italy) and the Shamouti (predominant in Israel). They are harvested in January and February and delivered in bulk to the processing plant either direct from the groves or as rejects from fresh fruit packing houses. At the plant the fruit is stored in silos until required. The silos have internal baffles to prevent too much damage to the lower fruit, from the weight of the fruit above. Just prior to extraction the fruit will be washed with water sprays and scrubbed with rotary brushes. Some makes of extraction machines operate best with fruit of specific diameter, so the fruit may be graded into different sizes before reaching the machine. The two most widely used extraction plants are the In Line machine produced by the Food Machinery and Chemical Corporation (FMC) of America, and the equipment made by the Brown Machinery Corporation, also of America.

The standard model of an FMC machine will handle some 300 fruit per minute. The fruit is fed into the front of the machine and directed into special cups. These cups are made up of radiating fingers, such that those of the upper cup will descend and intermesh with those of the lower cup. Both cups are fitted with circular cutters at their centres, the cutter in the lower cup is connected with a perforated strainer tube beneath the cup. A fruit will be fed into the lower cup, and as the upper cup descends upon it, circles of the peel are cut from top and bottom. The volume enclosed by the cup fingers now decreases, so that the lower disc of peel and the fruit interior are forced down into the strainer tube. An orifice tube now rises into the strainer tube, compressing the fruit interior so that the juice is expressed, and drains
away through the holes of the strainer tube. The residual pulp or "rag" is ejected from the tube and removed as waste. In the mean time the peel has been forced through an annular space surrounding the upper cutter to be rejected. As this happens the outer orange part of the peel (the flavedo) is abraded by the cup fingers to give "peel flakes" which contain the bulk of the essential oil from the fruit. The flakes are normally washed away by water sprays to a screw press, where an emulsion is squeezed out and then separated into essential oils and water using centrifuges.

The extractor built by the Brown Corporation utilises the more conventional reaming operation similar to those used in domestic kitchens. The fruit is fed singly past a vertical knife blade which slices the fruit into two. The fruit halves are then placed into cups so that their cut faces are horizontal and facing downwards, their flesh is removed by rotating reamers rising from beneath the cup, and the extracted juice drains away. The empty peels are ejected from the cups, and the oil rich flavedo layer is again removed as "peel flakes" by means of peel shaving blades and rollers. The peel flakes are also removed by water sprays and the oil extracted as previously described. Each of these Brown machines can handle between 300 and 400 fruits per minute.

The juice obtained from both of these machines is of so called single strength with a soluble solids of 11° Brix, and as such is comparable with juice obtained by hand reaming a fresh orange. The next stages of the juice processing will be dependant upon the type of final product required. A great deal of the juice obtained from Mediterranean or South African factories is exported to Europe, for use in compounded products which do not rely solely on the juice for their flavour. Also, legislation
in countries such as Western Germany which prohibits the use of artificial colours and clouding agents in fruit based beverages, induces the fruit processors to produce juices highly coloured and densely clouded in which flavour considerations may be secondary. Thus, the single strength juice is passed through screens and centrifuges to adjust the pulp content of the juice. It is then flash pasteurised to kill any contaminating microorganisms and eliminate natural enzyme activity. The juice is now evaporated using one of the many types of thermal machines to give concentrates of various densities. A six fold concentration of the juice is commonly practiced (Sinclair, 1961) this yielding a juice of $66^\circ$ Brix soluble solids. Such a six fold concentrate may be slightly diluted or "cut back" using fresh juice to a soluble solids of $44^\circ$ or $50^\circ$ Brix. Such products would then be either treated with preservative such as sulphur dioxide and packed in bulk containers, or hot filled into cans which are then sealed and spin cooled beneath water sprays. This concentrate may then be shipped to Europe for further processing into products. The nature of the products will be discussed in more detail at a later stage in this thesis.

In contrast to the concentrate produced for use on the European market, the American market favours a product which is consumed with only the addition of water. This product obviously relies on a processing which is capable of preserving the flavour of the fresh juice to a greater extent than the concentration process described above. The juice will have been extracted as described earlier in this introduction, but will have been centrifuged more efficiently to remove fruit solids before concentration. The evaporator used for this type of product is usually of the temperature accelerated short time type (designated TASTE). This is a falling film type of evaporator (Veldhuis, 1971) in which the dwell
time can be measured in minutes compared with the hours more commonly employed in other systems. At least one stage of the TASTE plant operates at 200°F to 212°F so incorporating a pasteurisation step. The juice will have been concentrated to 65°F Brix and then "cut back" to 45°F Brix by the addition of fresh juice. During the mixing of the "cut back" stage the product temperature will have been reduced to between 30°F and 40°F. Further cooling may be achieved by passing the juice through vottators before filling into packs, the most popular domestic pack is the 6oz container which reconstitutes to 24oz when three volumes of water are added. These packs are normally stored at 0°F until required for consumption.

A third type of citrus process which yields a product which is widely utilised on the British Market is the so called "Comminuted Base". The exact methods of base production are not readily obtained since they are regarded as highly confidential to the individual processing company. In general terms they consist of blending together juice, pith and peel of the fruit in a specified proportion. This mixture is then passed through mills fitted with carborundum grinding stones, and so comminutes or disintegrates the solid fruit constituents. It is considered (Robbins, 1973) that the disintegration process will release pectin from the peel which will form an emulsion in the presence of the juice and oil. The liquor from the mills is flash pasteurised at 95°C to eliminate pectinesterase activity, and then treated with a preservative such as sulphur dioxide and packed into casks or drums. These "bases" are then shipped to Great Britain and stored until required. In the production of bases at least two major types of orange product are recognised (Lampitt, 1972, personal communication). These are firstly, a process whereby the juice is
separated from the fruit and then mixed with the isolated peel and pith, and secondly where whole oranges are subjected to a comminution process.

It has proved impossible to obtain any more detail on this second process because of commercial confidentiality. In the experimental part of this report, the results obtained from an examination of both these types of commercial bases will be described.

Thus, it can be seen that basically three types of orange juice are produced by the processors. There is the concentrate intended for use in preparing various types of orange beverage; the frozen concentrate which is intended for dilution with water and then consumed, and finally the comminuted base which is intended for processing into various types of beverage. Of these three products the frozen concentrate is the most expensive, and the comminuted base the cheapest. In addition to its lower price, other advantages are claimed for bases (Robbins, 1973). These include greater flavour intensity, a greater clouding effect and a greater stability when processed into a product for domestic consumption.

The bases and the concentrates produced by heating are generally used to produce three main types of retail product. These are carbonated orange beverages, the orange drink and the squash. The orange drink and the squash are recognised as being two distinct products in terms of legislation and pricing. An orange drink must contain a minimum of 10% single strength comminute whereas a squash must contain at least 25% of a single strength juice (Soft Drinks Regulations 1964). In addition both of these products will contain sucrose, other sweeteners, preservatives, citric acid and colouring matter, and may contain additional essential oils and flavourings. (A detailed formulation is contained in the Material and Methods Section). The orange drink and the squash are
diluted with water before consumption.

A recent survey (Dieperink, 1973) on the importance of all types of soft drinks in the Common Market countries, indicated that of six beverages, Beer, Coffee, Milk, Wine, Soft Drinks and Tea the following preference ratings were obtained. In the Netherlands soft drinks were the third most consumed beverage. In the United Kingdom, Germany, Italy, and Belgium they rated fourth, whilst in France they were scored fifth. To maintain their position in the Market these beverages must have a high consumer appeal. This attraction will mainly centre upon the flavour and refreshing aspect of the drink, but will also to some extent be influenced by the appearance of the product. From the standpoint of appearance, it is probably the nature of the product on the shelf at the retailers which influences potential purchase as much as the appearance of the product which has been diluted for consumption. It is considered by the industry that the consumer requires a product of high cloudiness or body and with visible evidence that the product contains fruit tissue. Unfortunately, citrus beverages and orange products in particular are prone to separate into distinct layers during storage of the bottle. Thus, the would be customer is frequently confronted by a bottle in which the fruit debris and perhaps the bulk of the cloud have settled to the bottom of the bottle, and since the cloud has settled out, the bulk of the bottle consists of a rather weak, hazy orange red coloured solution and the neck of the bottle may contain either a substantial quantity of white opaque materials or a heavy ring of an oil. Severe cases of such product separation are definitely not at all attractive to the consumer, who is likely to purchase a product with a more pleasing and acceptable appearance.
Theories of Orange Cloud Stability

The body or cloud of orange juices is generally considered to be produced by the suspension of fine particles of fruit and oil droplets in an aqueous phase. Since, as mentioned above the cloud sometimes settles to the bottom of the pack and the oil to the top of the pack, it has been considered (Verbeck-Inckel, 1966) that a guide to the rate of separation may be given by Stokes' law; and this author presented a modified form of the law as follows:

\[ U = \frac{a r^2 \rho_1 - \rho_2}{\eta} \]

where \( U \) = the rate of travel of the disperse phase
\( a \) = a constant
\( r \) = radius of the disperse phase particles.
\( \rho_1 \) = specific gravity of the disperse phase
\( \rho_2 \) = specific gravity of the continuous phase
\( \eta \) = viscosity of the continuous phase.

Stokes' law is strictly only applicable to a system in which the freely settling particles are spherical, of a non porous incompressible nature and are suspended in an incompressible fluid. However, in orange juice it is likely that the fruit particles (which will be of degraded cellular origin) will be of various shapes and sizes, porous and compressible. Thus Becher (1965) considered that an extended version of Stokes' law would be applicable where the viscosity of the disperse phase should be taken into account. But this modified equation gave even greater rates of separation than those calculated from the Stokes' law equation cited above. Ranganna and Raghuramaiah (1970) have shown
that fruit particles of various sizes settled at similar rates, and it would therefore appear that factors not included in Stokes' law modify the sedimentation behaviour in the orange juice system.

However, even if Stokes' law did not accurately describe the rate of sedimentation it was considered that on the general basis of the law the rate of separation would be decreased by the following factors:

(a) Reduction in particle size.
(b) Reducing the density difference between the particle (dispersed phase) and the solute or continuous phase.
(c) Increasing the viscosity of the solute.

The particle size of the disperse phase will be related to processing history. The size of the particles which are permitted to remain in the juice will depend on how the "polishing" centrifuges are set, but normally most coarse pieces of tissue are removed. Some juices and most bases are subjected to an homogenization process in an attempt to reduce particle size. One major factor that has been used as a guideline by the industry is that the particle size should not be reduced below approximately 0.5μm (ie 5000Å), otherwise the particles would be smaller than the wavelength of light, and thus cease to reflect light and hence the product would not appear cloudy (Goodall, 1970). In general terms a mean particle size of about 1μm has been considered desirable for the tissue fragments and oil droplets (Shenton, 1972).

The density of the continuous phase or solution in a product will obviously be related to product formulation, but a typical figure for beverages available on the British market would be a density of 1.1
based on a formulation containing 25% by weight of sugar (Shenton, 1972). The density for the dispersed phase particles is very much more difficult to obtain, since the nature of the particles will be different. In the case of the tissue particles the density will be greatly influenced by the degree of occluded air bubbles, and no data has been published in this area. For the citrus oils, however, some information is available. The citrus oil contains 85-95% of d-limonene and density values of citrus oil approximate closely to that for d-limonene, giving a figure of 0.840 (Handbook of Chemistry and Physics 1969). Thus, it can be seen that in orange juice products one is endeavouring to suspend particles with a specific gravity in the range 0.84 in a solution of SG 1.1. In the past it was common practice for soft drink manufacturers to increase the SG of the oil by incorporating so called weighting agents. The most commonly employed weighting agents were brominated vegetable oils (BVO's) of SG 1.35 and these are blended with the citrus oils to increase the SG of the citrus oil to that of the suspending solution. However, the use of such brominated oils is no longer permitted, as it was considered that the cumulative effect of ingested brominated oil represented a health hazard (Statutory Instrument 1101, 1970). Other materials which have been tried as weighting agents have included rosin esters or sucrose esters. In the case of the rosin esters their SG is 1.07 and although this is greater than the citrus oils it is not as high as the BVO's and hence their efficiency as weighting agents is somewhat reduced (Goodall, 1970).

Numerous attempts have been made to increase the viscosity of the disperse phase. The extent to which the viscosity of the solution can be increased by the addition of sucrose is very severely restricted by the flavour considerations of the product. Too much sugar produces a sickly
sweet unacceptable beverage. Thus, most methods to increase viscosity have utilised polysaccharide thickeners or gums. They include alginates, carrageenans, furcellaran, gum arabic and locust bean gum (Goodall, 1970). Dakin (1953) showed that the modified alginate propylene glycol alginate was most effective in suspending natural fruit tissues. It is considered (Glicksman, 1969) that these materials, whether of the natural or modified type, are effective in increasing the viscosity of the aqueous solution as a result of their hydrocolloid nature, ie the individual colloidal particles absorb water and swell, so increasing the viscosity of the solution.

In addition to the concepts based on the application of Stokes' law, several workers have applied the theory of emulsions to endeavour to understand the factors controlling the stability of citrus juices. These have included adsorption theories, considerations of interfacial phenomena, and electrical theories and these have been discussed by Becher (1965) and Summer (1954).

On the basis of adsorption theory, the stability of an emulsion may be attributed to the adsorption of an emulsifying agent on the surface of the suspended globules. In simple terms, an emulsifying agent is considered to consist of molecules with a polar group at one end, and a non polar group at the other end. Thus, it would be expected that when an emulsifier is added to an orange juice the non-polar end will become attached to the oil droplets leaving the polar end exposed in the aqueous phase. Whence, oil droplets become surrounded by polar groupings of similar charges, are mutually repelled from each other, thus tending to stay apart and in suspension.
The main use of emulsifiers in citrus drinks has probably been in connection with additional essential oil which is often added to improve flavour (Goodall, 1970). Here the essential oil will be formed into an emulsion in the presence of a suitable emulsifier, and then added to the product. The choice of emulsifier will be dependant upon a number of factors, and the actual systems which are used are regarded as confidential information.

The theories which involve interfacial phenomena have endeavoured to obtain information about the properties of interfacial films and their role in stabilising emulsions. But the results obtained in this field are far from complete and so its application to orange juice is little understood.

Electrical theories may be considered to be an extension of the adsorption and interfacial theories of emulsion stability as mentioned above. Mizrahi and Berk (1970) measured the charge on cloud particles, in orange juice, and found that all the particles were negatively charged, and that this charge decreased with decreasing pH from 5.1 down to pH 3.4. However, they concluded that in relation to cloud stability the hydration state of the particles was more important than their electrical charge.

The most generally held view regarding orange juice cloud stability assumes that the cloud particles are stabilised by suspension in a soluble pectin media (Rouse, Atkins 1955) and that factors which favour pectin gel formation also increase cloud stability. The thermal pasteurisation of orange juice which inactivates pectic enzymes has been shown to enhance cloud stability (Bissett, Veldhuis, Gnyer and Miller, 1957, Moore, Rouse and Atkins, 1962). The majority of the published literature
on the stability or clarification of orange juices has been centred upon the topic of citrus pectin or the pectinolytic enzymes. The extensive work in this area has been reviewed by Joslyn and Pilnik (1961).

The term pectin covers a group of complex colloidal carbohydrates that contain a high percentage of anhydrogalacturonic acid units, which are thought to be combined into a chain like configuration through O-1-4 linkages (Doesberg, 1965). On the basis of viscosity measurements, it has been concluded, that in the case of pectin from orange tissues, some 200 to 300 galacturonic acid residues are probably present in the chain (Sinclair, 1961). The carboxyl groups of the galacturonic acid units may be partially esterified by methyl groups and some of the hydroxyl groups may be acetylated. Doesberg (1965) considers that non-uronide constituents may also be present either in the chains or attached as side chains. Pectic substances show a very great heterogeneity as result of the variation in the amount and distribution of methoxyl, acetyl and non-uronide constituents. The molecular weight of pectic substances may vary from approximately 10,000 to 200,000, the low molecular weight substances being water soluble, whilst the higher molecular weight compounds are insoluble in water (Kertesz, 1951).

For analytical purposes pectin may be classified into the following forms:

(1) Water soluble pectin, extractable by water.

(2) Acid soluble pectin, extractable by dilute hydrochloric acid from a water insoluble fraction.

(3) Calcium pectate and pectinate, extracted in the presence of a calcium binding agent such as oxalate.

(4) Protopectin, extracted by sodium hydroxide.
In addition, the water soluble pectin is further divided on the basis of the degree of esterification. The low methoxy pectins (LMP) are less than 50% methylated, whilst high methoxy pectin (HMP) have degrees of esterification greater than 50%.

Citrus fruits are a very rich source of pectic substances (Sinclair, 1961). The distribution of total and water soluble pectin in the components of oranges are listed below in Table 2 (Nanji and Norman, 1928).

**TABLE 2**

<table>
<thead>
<tr>
<th></th>
<th>Soluble Pectin %</th>
<th>Total Pectin %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peel</td>
<td>7.5</td>
<td>15.9</td>
</tr>
<tr>
<td>Albedo</td>
<td>18.5</td>
<td>38.8</td>
</tr>
<tr>
<td>Pulp</td>
<td>10.5</td>
<td>12.4</td>
</tr>
</tbody>
</table>

Orange pectin is approximately 50-90% esterified (McCready et al, 1951) and of very low acetyl content (Kertesz, 1951). The molecular weight of orange pulp pectin is about 150,000 (Sneider and Bock, 1937) whilst that of orange juice is about 50,000 (Svedberg and Grimel, 1938). The level and type of pectin material present within the orange varies during the course of development of the fruit (Sinclair and Jolliffe, 1961). Thus, Gaddum (1934) has reported that in Valencia oranges the total pectin content of the albedo decreases by some 35% during the last two months of growth, and that of the pulp by some 20% during the same period. The pectin content of commercially available bases has not been published, but it is estimated to be between 0.004 and 1.0% (Shenton, 1972).
As was mentioned above it has long been established that heat pasteurisation of orange juice increases the stability of the cloud. Since the appearance of a clarified citrus juice frequently showed the presence of a gel like material, it was concluded that a soluble pectin matrix was necessary for stability and that heat treatment inactivated a pectinolytic enzyme. The only pectinolytic enzyme so far identified in orange juice is pectin esterase (PE) (Sinclair, 1961). This enzyme was first isolated and characterised in orange juice by MacDonnell, Jansen and Lineweaver (1945). Pectinesterase acts by hydrolysing ester groups of pectinic acid, producing methanol and free carboxylic acid groups, and yielding pectic or pectinic acids with a lower degree of esterification. Hydrolysis is confined to methyl ester groups adjacent to free carboxylic acid groups and will then proceed linearly along the chain. MacDonnell et al (1945) found that the activity of PE is increased in the presence of salts, particularly divalent cations such as calcium. Maximum enzyme activity occurs at pH 7.5, but the activity is still quite considerable at the lower pH's encountered in juices and bases. Orange pectin esterase activity differs widely in the various anatomical elements of the fruit, the greatest activity being observed in the juice sacs (Rouse, 1953). The distribution of activity in the various tissues of the Valencia orange is given in Table 3.

**TABLE 3**

| Pectin esterase activity (P.E.u.) g x 10³ (on a dry solids basis) |
|-----------------|----------|
| Peel            | 34.4     |
| Albedo          | 20.7     |
| Juice Sacs      | 474.3    |
| Juice           | 1.9      |
Thus, when juices contain great amounts of pulp (i.e., juice sac residue) more vigorous pasteurisation processes are required to inactivate the enzyme. Rouse and Atkins (1952) have shown that in order to achieve 100% enzyme inactivation in a Valencia orange juice, containing 10% pulp, it was necessary to heat to 210°F (98.9°C) with a holding time of 10 seconds.

Dietz and Rouse (1953) considered that the mechanism of juice clarification involved the action of pectin esterase on juice soluble pectin, converting it to low methoxyl pectin, which then reacted with polyvalent cations to form insoluble pectates. Thus, the concentration of juice soluble pectins, which was considered necessary for particle suspension, had been depleted, and the precipitation of the insoluble pectates was presumed to occlude the cloud particles and remove them from suspension.

However, it has been shown by Baker and Bruemmer (1969) that soluble pectin was not necessary for cloud support, as a stable suspension of orange juice particulates could be made in water. Furthermore, Baker and Bruemmer (1972) have been able to produce a stable orange juice cloud by treating juice with a commercial pectinolytic enzyme, thus virtually eliminating the heat pasteurisation stage. From their studies using commercial pectinolytic enzymes on orange juices, they have concluded that it is the formation and precipitation of insoluble pectates which results in the clarification of orange juice. The role of commercial pectinolytic enzymes in stabilising orange juice is considered to be as follows. Commercial samples of pectinolytic enzyme contain in addition to pectin esterase, polygalacturonase (PG) and polymethylgalacturonase (PMG). The other member of the family of pectic enzymes pectin trans eliminase (PTE) has not been detected in commercial enzymes from higher plants (Ishii and Yokotsuka, 1971). Baker and Bruemmer (1972) considered that it
it is the ratio of the polygalacturonase to polymethylgalacturonase that
is the important factor in the action of the commercial enzymes. The
degradation of pectin by pectin esterase, polygalacturonase and polymethyl-
galacturonase may proceed via the scheme proposed by Demain and Phaff
(1957) which is depicted in Fig. 2.

Whence it can be seen that the ultimate catabolism products from juice
soluble pectin, will be either, low methoxyl pectin, or soluble oligo-
galacturonic acids. Thus in orange juice, the route via natural pectin
esterase takes place to produce low methoxyl pectin which precipitates
with polyvalent cations and so clarifies the juice. Treatment of juice with
a commercial enzyme with a high polygalacturonase to polymethylgalacturonase
ratio, produces a greater proportion of soluble digalacturonic acid than
low methoxyl pectin. So that, the mechanism of clarification via precipitation
of low methoxyl pectin is minimised. Pilnik and Varagen (1970) have reported
that orange juice contains no detectable polygalacturonase or polymethyl-
galacturonase.

So, the useful role of pectin in stabilising orange juice cloud has
been brought into doubt. However, orange juice does contain a large amount
of pectin, and it is clear that either thermal pasteurisation or controlled
enzymatic treatment are essential to obtain a stable cloud.

Nature of Cloud Particles
(a) Chemical

It is now pertinent to consider what is known about the nature of the
cloud particles. Chemical analytical techniques have indicated that the
cloud is composed almost exclusively of pectin, protein and lipid (Scott,
Kew & Veldhuis, 1965, and Baker and Bruemmer, 1969). The early data of
Scott et al (1965) suggested that the amount of these 3 major constituents
was pectin 60%, protein 1%, lipid 25%. However, Baker and Bruemmer (1969)
Protopectin

PG or PMG

Soluble pectin

PMG

Short chain soluble pectin

Low methoxyl pectin

PE

M++ Insoluble pectates

PE

PG

Short chain low methoxyl pectin

M++ Insoluble pectates

PG

Soluble oligogalacturonic acids

PG = Polygalacturonase
PMG = Polymethyl galacturonase
PE = Pectin esterase
M++ = Bivalent cation

Fig. 2. Schematic representation of the degradation of pectin.
considered that method of pectin analysis used by Scott et al was inaccurate, and their own results for these constituents were pectin 34%, protein 34% and lipid 25%. Furthermore, Scott et al also considered that the composition of the cloud was quite different from that of the other anatomical elements of the fruit, and so the cloud should be regarded as a distinct anatomical component of the fruit and not merely as small fragments of pulp.

Recent spectroscopic investigation of an alcoholic extract of the cloud from Valencia oranges, has indicated absorption maxima in both the visible and ultraviolet radiation ranges (Petrus and Dougherty, 1973). These authors have shown that this alcoholic extract contains carotenoids, polyphenols, flavanoids and ascorbic acid.

b) Microscopy

The examination of the cloud particles by microscopical techniques utilising both the light and electron microscopes has greatly aided the identification of some of the cloud particles. Mizrahi & Berk (1970) using a combination of light and electron microscopy, on the cloud from Shamouti oranges identified chromoplastids, fragments of pulp and rag, oil droplets and needle like particles which they identified as the flavanone hesperidin. Resch and Schara (1970) have made extensive studies using light microscopy of the anatomical elements of the orange, and the particulate material found in orange juices and commercial products. They have recognised all the particles described by Mizrahi and Berk, and have also described the nature and probable origin of several of the pulp and rag particles.

Microscopical Structure of the Orange

The anatomical elements of the orange are composed of the following tissue types. The exocarp comprises the orange coloured so called
flavedo. The outer epidermal cells of this layer have a waxy cuticle. Beneath the epidermal cells are found the oil glands which contain the bulk of the essential oil that is found in oranges. The other layers of the exocarp contain isodiametric cells which frequently bear the coloured chromoplasts and calcium oxalate crystals.

The mesocarp or albedo is found lying immediately beneath the flavedo. It comprises of tube like cells with very thick cell walls which are separated by large intercellular spaces containing air. It is the combination of these large air spaces and thick cell walls which impart to this tissue its characteristic yellow/white colour. The mesocarp also contains numerous lignified vascular strands. The spongy parenchyma cells frequently contain characteristic clusters of hesperidin crystals in the shape of spherulites. The tissue is also particularly rich in pectin. Flavedo and albedo together comprise the region that is more commonly called the peel.

The endocarp is the edible portion of the fruit and consists of a number of carpels which are separated by carpellary wells. Within each carpel are found the juice vesicles or sacs and the seeds (except in certain seedless varieties). The carpellary or segment walls contain both mesocarp and endocarp tissues and their cells may contain many calcium oxalate and hesperidin crystals. The juice sacs consist of an outer epidermal layer which contains a cuticle. These epidermal cells are long and run in the direction of the juice sacs. The cells beneath this epidermis are large rounded cells which contain the orange juice and chromoplasts. According to Davis (1932) the central cells of the juice sac contain oil droplets. Some of the subepidermal cells contain crystals which will be either of calcium oxalate or hesperidin.
The final anatomical element which can be recognised is the central column or core. This tissue is comprised of the albedo type of cells which form into distinct vascular strands.

Most of the microscopical investigations which have been undertaken to examine the particles in orange juices and products have been performed with the aid of the light microscope. Although these studies have proved most useful, they have of course been limited by the fact that the resolution of the light microscope is of the order of 0.2 μm and thus an unequivocal identification of particles smaller than 0.5 μm becomes difficult, and particles smaller than 0.2 μm would not even be detected. The electron microscope operating at some 100 keV has a theoretical resolution of less than 0.1 nm and a practical resolution of better than 1 nm (Meek, 1970). The potential to be gained from examining citrus products with the aid of the electron microscope has been clearly demonstrated by Mizrahi and Berk (1970) who have shown that particles smaller than 0.2 μm may be detected in the cloud from orange juice. However, their studies only utilised one method for preparing the samples and that was the drying down of a suspension of juice particles onto a carbon film on a support grid. Although this is a perfectly valid technique, it can only yield a somewhat limited amount of information about the particles which may have also been subjected to gross distortion during the drying operation. A study which applied a wider range of specimen preparative procedures might have given more useful information.

Specimen Preparation for Electron Microscope

a) Thin Sectioning

One of the most widely adopted techniques for studying the structure of biological specimens is the examination of thin sections of the tissue. In order to prepare suitable thin sections for examination in the electron microscope it is normally necessary to embed the sample in a supporting medium. Prior to embedding the tissue it would normally be chemically fixed in reagents such as buffered glutaraldehyde (Sabatini, Bensch &
Barrett, 1963), or buffered osmium tetroxide (Palade, 1952). The fixed samples are then dehydrated (most normally by passing through a graded ethanol series) and then infiltrated with and embedded in a suitable resin. Numerous resins have been proposed such as Araldite (Glauert, Rogers and Glauert, 1956) methacrylates (Newman, Borsky, Swerdlow, 1949), and Epon (Finck, 1960). Resins which have a low viscosity such as Maraglas (Freeman, Spurlock 1962) and Spurr's resin (Spurr, 1969) have been found to be particularly suitable for plant tissue. Thin sections of the tissue (some 10-50 nm thick) are cut on an ultramicrotome equipped with either a glass or diamond knife. The scheme outlined above may be regarded as the conventional approach to thin sectioning, and departures from this approach have been suggested by several authors. Thus, Peterson & Pease (1970) utilise a mixture of aldehydes and urea as a combined fixation, dehydration and embedding system, which they claim minimises the loss of lipid material from the tissue. The necessity to embed in plastic resins can be eliminated by using the cryoultramicrotomy methods that were introduced by Bernhard & Leduc (1967). Some workers (Bernhard, 1972; Bauer, Sgarlakie 1972 and Sjostrom, 1972) incorporate a light fixation step prior to freezing and sectioning the tissue. Whilst Appleton (1972) and Christensen (1969) do not even fix the tissue but solely freeze the sample and then cut sections. The results obtained to date from cryoultramicrotomy have been limited to studies on animal tissues or yeast and although they are very interesting and show great promise it is allegedly a very difficult technique which is still in its infancy.

b) Freeze etching

The traditional thin sectioning method may be criticised as subjecting tissues to a variety of drastic chemical pretreatments, which may significantly alter the true cellular structure. The cryomicrotomy techniques endeavour to minimise the chemical pretreatment as does the freeze etching technique which was introduced by Steere (1957). This procedure involves the rapid snap freezing of a sample in a coolant such as liquid Freon to
-150°C. The sample is then fractured, perhaps subjected to an etching stage, and then a replica of this freshly exposed surface is prepared. The fracturing, etching and replication stages are carried out under vacuum in a modified vacuum coating apparatus, and the first commercially available freeze etch machines were based on the work of Moor et al. (1961).

Although, the chemical pretreatments are minimised in freeze etching, the technique is not completely free of artifacts, and these fall into three broad categories. Firstly, damage due to ice crystal formation during the freezing stage (Moor, 1964). Secondly, structural changes may be brought about by deformation of the sample during the fracturing process (Clark, Branton, 1968, Dunlop and Robards, 1972). Thirdly, changes in the appearance of the sample surface caused by deposition of contaminants from the vacuum system (Dreamer, Leonard, Tardieu and Branton, 1970; Staehlin and Bertaud, 1971).

The problem of ice crystal formation can be minimised by adopting two approaches to specimen preparation. In many instances the sample may be pretreated with an antifreeze or cryoprotectant prior to the freezing step. Several cryoprotectants have been proposed, of which glycerol and ethylene glycol are the most widely adopted (Moor, 1964; Moor, 1969), and both of these systems minimise the growth of ice crystals. The second approach involves increasing the rate of freezing of the sample, so that again ice crystal growth will be minimised. Riele (1968) has shown that high pressure freezing of very small samples virtually completely eliminates ice damage, but the technique is difficult and at present beyond the scope of all but a well equipped cryogenics laboratory. An increased rate of freezing may also be achieved by freezing very small samples of the tissue, and techniques for rapidly freezing small droplets of a tissue
suspension have been developed by Bachmann and Schmitt (1971), and Buchheim (1972). Even so, despite the methods listed above some samples are still very prone to damage by ice formation, and considerable attention must be paid to this possibility when interpreting the results obtained from freeze etching.

The deformation of the specimen during the fracturing process is a problem which cannot be readily overcome. But in many cases the appearance of the replica of the sample indicates that some deformation has occurred.

The third problem of the deposition of contaminants on the sample surface, which may be subsequently interpreted as being the genuine structure of the sample, can be alleviated by ensuring that the vacuum system is perfectly clean and adopting the recommended methodology to minimise such contamination (Dreamer et al 1970).

The freeze etching technique is being utilised to both complement and in many instances supercede thin sectioning. It is being used to study plant tissues (Moor & Muhlethaler, 1963, Northcote & Lewis, 1968, Robards, Austin & Parish (1970). It is an ideal technique for studying membrane structures (Staehlin 1968; Dreamer et al 1970; Weinstein & McNutt, 1970). Freeze etching is also being widely adopted to study particulate suspensions of virus (Abram & Davis, 1970; Nermut & Frank, 1971) bacteria and yeast (Moor & Muhlethaler, 1963; Bayer & Remsen, 1970) and non biological polymers (Reed & Barlow, 1971 and Dunlop and Robards, 1972).

Negative Staining

The third major preparative technique which is used for the examination of biological tissues by transmission electron microscopy is the negative
staining procedure, introduced by Brenner and Horne (1959). The technique involves preparing a suspension of the sample in a solution of an electron opaque salt, such as a phosphotungstic acid. A drop of this suspension is then dried onto an electron microscope grid that is covered with a thin film of carbon or plastic. When this grid is viewed in the electron microscope the particles of the sample are rendered visible by their contrast against the surrounding electron opaque salt. In addition to phosphotungstate several other salts may be utilised, these include ammonium molybdate, silver nitrate, sodium tungstate and uranyl acetate or nitrate (Valentine and Horne, 1962).

The negative staining technique has the advantage over both thin sectioning and freeze etching, that with suitable specimens very fine structural resolution can be achieved. With thin sections it is very difficult to obtain resolution of better than about 5 nm. Freeze etching improves on this figure and can yield resolution down to about 2 nm, but at this level the size of the grain of the heavy metal used to prepare the replica becomes a limiting factor. However, with negative staining it is possible to achieve resolution of the order of 1 nm.

A wide number of materials have been studied by negative staining, and of these probably one of the most significant applications has been in the field of virology (Horne and Wildey 1963, Nermut 1972). Here it has been used both to elucidate structure and as a diagnostic aid.

Other systems which have been studied have included bacteria (Glauert, Kerridge and Horne, 1963) sub cellular organelles (Gray and Whittaker, 1960) and isolated protein molecules from sources such as muscle (Huxley, 1963) or collagen (Hodge & Schmitt, 1960). On the botanical side cellulose fibres isolated from the cell wall were studied by Heyn (1966, 1969).
Quantitative information concerning the number of particles present in a test suspension can be elucidated by the incorporation of a known concentration of marker particles such as latex (Watson, Russell and Wildy; 1963; Monroe and Brandt, 1970).

d) Scanning Electron Microscopy

In addition to the results obtained by the examination of materials with the transmission electron microscope, considerable information can be gleaned by examining the surfaces of the sample with the scanning electron microscope. In conventional scanning microscopes it is normally a prerequisite that the specimen is completely dry and coated with a thin conducting layer of a metal such as gold or silver. For many biological specimens the rigours of the drying and metal coating under vacuum can result in collapse and distortion of the cellular structure. With some specimens the damage caused by the drying stage can be minimised by using the critical point method of freeze drying (Robards, Crosby & Sharper 1974). The fairly recent development of freezing stages to be used on scanning electron microscopes has shown that some biological materials can be examined after merely rapidly freezing the sample (Nei et al., 1972) and that tissue preservation is good.

Electron Microscopy applied to Foodstuffs

The application of such electron microscopy techniques to study samples of foodstuffs has only been practised on a somewhat limited basis, so that the literature on the structure of food materials is somewhat sparse.

The appearance of meat has been described by, amongst others Voyle (1969) Davey and Dickson (1970) and the changes produced in meat structure by curing with salt has been discussed by Lewis and Jewell (1972). A wide number of foods that contain a relatively high percentage
of lipid have been studied, and these have included cheese (Reed, 1969), butter (Wortmann, 1965), chocolate (Jewell, 1972), ice cream (Berger, Bullimore, White and Wright, 1972) milk (Buchheim, 1969) and other vegetable or animal fats (Jewell and Meara, 1970). On the botanical side the structural changes produced during the freezing of vegetables has been discussed by (Bassi & Crivelli, 1968, 1969), and the structure of pickled vegetables such as cauliflower was described by Saxton and Jewell (1969) and of onions by Jewell (1972). Several authors have described the structure of starch granules, as seen by electron microscopy techniques, both before and after processing such as linterisation (Sterling & Spit, 1958, Leonard & Sterling, 1972).

**Objective of Research**

Finally, it is a technological observation that orange juices produced from different geographical locations, or from the same area but processed using different equipment, frequently give rise to products which exhibit marked differences in cloud level. The manufacturers do not understand all the factors which control the cloud level within the juice. Therefore it was considered pertinent to undertake a more thorough investigation into the nature of the particles in orange juice, using a wide range of electron microscopy techniques.
MATERIALS AND METHODS

Two cultivars of oranges and a range of juices, bases and products have been examined during the course of this investigation. The oranges were purchased from a local fruiterers and the observations were made on either Spanish Navel's or Israeli Jaffa's. The juices, bases and products were obtained from several commercial sources. The juices are classified according to their country of origin and their soluble solids content. The soluble solids are expressed as degrees Brix, where a 10% w/v aqueous solution of sucrose would be 10° Brix. The following range of samples has been examined:

**Juices**

(a) Israeli 11° Brix.
(b) Israeli 44° Brix.
(c) Israeli 66° Brix. (Supplier 1)
(d) Israeli 66° Brix. (Supplier 2)
(e) Spanish 55° Brix.
(f) South African 66° Brix.

**Bases**

(a) Spanish 200%.
(b) Spanish 30° Brix.
(c) Israeli 30° Brix. (Supplier 1)
(d) Israeli 30° Brix. (Supplier 2)
(e) Israeli 30° Brix. (Supplier 3)

**Products**

The products had been prepared using the following bases:

(a) A mixture of Israeli High and Low cloud bases.
(b) An Israeli High Cloud base (used in (a)).
(c) An Israeli Low Cloud base (used in (a)).
(d) A Spanish Low Cloud base.
(e) An Italian Low Cloud base.
(f) A product containing a Blend of Spanish and Israeli bases.
The exact formulation of the products was not disclosed, but a general recipe which could be used to produce a product is given in Appendix I.

In addition a limited range of lemon juices has also been examined.

(a) High Cloud Sicilian 400. (1972 season.)
(b) Normal Sicilian 400.
(c) New High Cloud Sicilian 400.
(d) High Cloud Sicilian 400. (1973 season.)

Isolation of Juice from Fresh Fruit

Fresh fruit were cut in half in a plane at right angles to the central core, then each half was hand reamed on a kitchen type fruit reamer. The liberated juice was then filtered through a nylon sieve (1mm square mesh) and the filtrate retained for use.

An alternative method of obtaining juice involved the withdrawal of the juice from individual juice sacs using a syringe. The fruit was first peeled and then the segments separated to expose the juice sacs. A small needle fitted to a syringe was then carefully inserted into the juice sac and the contents of the sac withdrawn and retained for use.

Microscopy Methods

(1) Light Microscopy
(a) Fresh Tissue

The various anatomical elements of the oranges were dissected out and placed in formalin, acetic acid, alcohol fixative (F.A.A.
70% alcohol 90ml, glacial acetic acid 5ml, formalin 5ml) for 24 hrs. The fixed tissue was either dehydrated by passing through a graded ethanol series and embedded in paraffin wax, or quick frozen in liquid nitrogen prior to cutting frozen sections. The tissue embedded in wax was sectioned at 8 - 10μm using a Reichert Sledge Type microtome. The sections were harvested onto slides, and then the wax was removed with xylene. The frozen tissues were sectioned using a Slee Pearse Cryostat operating at -25°C and sections of 8 - 10μm were again collected onto glass slides.

The sections were stained with Safranin (1% Safranin in 95% alcohol) for 2 hrs. and fast green (1% aqueous for 5 mins. and then viewed with bright field illumination. Photomicrographs were prepared on Ilford FP4 film which was developed in Promicrol, or using Kodak Ektachrome colour film.

Some of the sections were examined without staining but using either phase contrast or polarised light forms of illumination.

(b) Juices, Bases and Products

These materials were examined by placing a drop of the solution on a glass slide, covering with a cover slip and viewing with either phase contrast or polarised light.

Prior to examination the juices and bases were normally diluted with distilled water to give a solution equivalent to that of single strength orange juice, i.e. 11° Brix. The diluted materials were then centrifuged at 360xg for 10 mins in order to achieve a degree of fractionation (Resch and Schara, 1970). During the course of this investigation other levels of centrifugation were tested to see
if 360xg was the optimum force for separating coarse particles. The details are included in Appendix II, but it was concluded that 360xg was probably optimum and also had the advantage of being the method most widely applied in the literature.

(2) Electron Microscopy

During this study three preparative methods, for transmission electron microscopy, have been utilised; they have been thin sectioning, negative staining and freeze etching.

(a) Thin Sectioning

In order to be able to embed and thin section the juices, bases and products, it was first necessary to encapsulate them in agar using the method introduced by Salyaev(1968). This method involves partially filling a pasteur pipette with the test liquid (juice, base or product) and then dipping the capillary end (1.5mm diam.) of the pipette into a warm solution of aqueous agar (3% w/v) to a depth of approximately 1cm. The pipette is then withdrawn from the agar, and the adhering agar allowed to set around the capillary tube. By careful manipulation of this set agar between the fore finger and thumb, the agar can be rolled nearly off the capillary tube so that it forms an agar cylinder but is still attached to the end of the capillary tube (see Fig. 3). The test liquid can then be dispensed from the pipette to fill the agar cylinder. The cylinder is then carefully removed from the capillary tube and the open end sealed with a drop of molten agar. This procedure yields small capsules of agar containing the test liquid.

The anatomical elements of the fruit were sliced into cubes of less than 1mm dimensions, with the exception of the juice sacs which
a. Pasteur pipette full of juice dipped in molten agar  
b. Pipette removed and agar allowed to set  
c. Microcapsule manipulated off pipette by use of finger and thumb. The microcapsule is filled by discharge from the pipette. Then fully removed and sealed with agar.

Fig. 3. Schematic representation of the preparation of agar microcapsules according to the method of Salyaev (1968)
were either kept intact or were emptied by piercing with a needle. These anatomical elements and the filled agar capsules were then fixed in phosphate buffered 3% (v/v), glutaraldehyde pH 7.2 for 4 hrs at +4°C (Sabatini, Bensch & Barnett, 1963), rinsed in distilled water and then post fixed in osmium tetroxide solution pH 7.2 for 24 hrs at +4°C (Palade, 1952). The fixed material was then rinsed in distilled water and dehydrated by passing it through a graded ethanol series (20 to 100% ethanol). The dehydrated samples were then infiltrated with and embedded in either a mixture of methyl and butyl methacrylate (50:50 v/v) or Maraglas (Freeman & Spurlock, 1962). The methacrylate resin was polymerised at 40°C for 24 hrs and the Maraglas at 60°C for 24 hrs.

The embedded material was sectioned using a Cambridge Huxley Mk I ultramicrotome equipped with a glass knife. The sections which were some 50 – 80 nm thick, were harvested onto collodion covered 100 mesh copper electron microscope grids. The method of covering the grids is described in Appendix 3. The sections were either viewed unstained or after staining with aqueous uranyl acetate (1% w/v) and lead citrate (Reynolds, 1963).

(b) Negative Staining

A range of negative stains were investigated initially to determine which staining agent and conditions appeared to give the optimum information. The stains which were evaluated were as follows:

(a) 2% aqueous phosphotungstic acid (PTA) at pH 1.0
(b) 2% aqueous PTA at pH 5.0 (adjusted with sodium hydroxide solution (1N)).
(c) 2% aqueous PTA pH 7.0 (Neutralised with sodium hydroxide (1N)).
(d) 2% aqueous uranyl acetate pH 6.0.
(e) 2% aqueous ammonium molybdate pH 6.0.

A standard procedure for negative staining has been utilised throughout this investigation. To 1 ml of the suspension under examination was added 1 ml of the appropriate negative stain, and to this was then added 0.5 ml of an aqueous suspension of polystyrene latex spheres of 0.375 μm diameter. These solutions were then mixed and a drop of the solution placed on a collodion covered 100 mesh copper electron microscope grid. The excess fluid was drained away with a filter paper and then the grid allowed to dry, before viewing in the electron microscope.

(c) Freeze Etching

The juices, bases and products were examined by freeze etching. The single strength juices (11° Brix) and the products were treated with glycerol prior to freeze etching, this was achieved by making the sample 30% with respect to glycerol. The concentrated bases and juices were not treated with glycerol.

The samples were prepared by placing a small drop (2mm diameter) of the test solution on a freshly formed and cleaned copper freeze etching stub. The stub and sample were then rapidly cooled by plunging into liquid Freon 12 at -150°C. The stubs were then transferred to liquid nitrogen and stored until required, this storage period did not normally exceed 2 hrs.

A frozen stub was placed on the stage of the NGN FE 600 freeze etching machine, the stage had been precooled to -140°C. A vacuum was then drawn and when a pressure of $2 \times 10^{-6}$ torr had been achieved,
the sample was warmed to -100°C. The sample was then planed and fractured using a microtome knife which had been cooled to ~190°C. Following the fracture of the sample the knife was kept positioned immediately above the sample in order for etching of the surface to occur. Etching was performed for periods of between 2 and 6 minutes. After the etching stage, the knife was removed from the position above the sample surface. A carbon platinum replica of the freshly fractured and etched surface was prepared by evaporating a layer of carbon platinum onto the surface from an angle of approximately 45° to the surface of the sample. The carbon platinum layer was then strengthened by evaporating a layer of carbon onto the surface of the sample.

The stub was then removed from the freeze etch unit and immersed in water to release the replica from the sample. The replica was then cleaned by passing it through a series of graded hydrochloric acid solutions (30, 70 and 100%) for ½ hour in each solution. The replica was finally rinsed in distilled water and picked up onto a copper electron microscope grid.

All of the samples prepared for transmission electron microscopy were examined with an Hitachi HS-7S electron microscope operating at 50 Kv, images were recorded on Ilford N50 emulsion, which was developed in Teknol developer.

(d) Scanning Electron Microscopy

Some samples of juice sacs and some of the material isolated from the cloud were examined using the scanning electron microscope. The samples were freeze dried onto Cambridge pattern stubs and
rotary coated with a layer of palladium or carbon, in an Edwards vacuum coating unit.

The stubs were examined in a Cambridge Instruments SIIA scanning electron microscope operating at 20 Kv. Some of the carbon coated samples were subjected to X-ray microprobe analysis whilst in the microscope. The samples were irradiated at 20 Kv and the X-rays detected using two gas flow proportional counters equipped with mylar windows.

PHYSICAL AND CHEMICAL METHODS

1. Determination of Soluble Solids

A refractometer method was used to determine the soluble solids of the juices, bases and products. A drop of the test solution was placed in an Abbe refractometer which had the specimen chamber water jacketed at 20°C. The soluble solids were then read directly off the scale calibrated for sucrose equivalent and expressed as degrees Brix.

2. Determination of Cloud Level

The cloud level of the various solutions was assessed by measuring their light absorbance at a specified wavelength using an Eel absorptiometer. Nephelometry was also evaluated as a means of measuring cloud level, but was abandoned because it proved unreliable (see Appendix 4).

The readings of the absorptiometer were standardised against suspensions of known amounts of bentonite in distilled water, according to the recommendations of Senn, Murray and O'Connor (1955).
The sample under test was diluted, where appropriate, with distilled water to give a solution of 11° Brix. This solution was then centrifuged at 360 x g for 10 minutes, and the supernatant layer, which was defined as the cloud, was retained. The supernatant was diluted further with distilled water 1 part sample to 3 parts water and placed in a glass Eel cell of 1 cm path length. A blank of distilled water and the test sample were placed in the Eel and the wavelength was adjusted to 660 mµ. The instrument was adjusted so that the blank read zero on the photoextinction scale of the galvanometer, i.e.: zero absorbance or 100% transmittance. The test sample was then brought into the light path, and the reading on the scale noted.

With the bases and some of the exceptionally cloudy samples which were examined during the last year of the project it was necessary to dilute the supernatant more than 1:3 with water in order to obtain readings of less than 100% absorbance.

3. Characterisation of a Pure Sample of Hesperidin

A sample of the flavanone hesperidin (whose structure is depicted in Fig. 4), was obtained from Koch-Light, and recrystallised from formamide/water according to the method of Pritchett and Merchant (1946). The recrystallisation involved preparing a 10% solution of hesperidin in formamide and heating together with activated charcoal, to 60°C for 30 mins. The solution was filtered, whilst warm, through diatomaceous earth. To the filtrate was added an equal volume of distilled water, and the solution was allowed to stand until crystallisation had occurred. The crystals were then filtered off and air dried. The recrystallisation was
Fig. 4. Structure of hesperidin.
repeated until crystals of constant melting point were obtained.

(a) Melting Point Determination

The recrystallised hesperidin crystals were very small, so their melting point was determined using a light microscope equipped with a melting point hot stage.

A sample of the crystals was placed on a cover slip, which was then inserted into the heating chamber of the melting point apparatus. The microscope was then adjusted to bring the crystals into focus, and a heat and infra-red absorbing filter was then placed between the heating stage and the microscope objective. The function of the heat filter was to protect the lower elements of the microscope objective lens, whilst the infra-red absorbing filter is designed to minimise the quantity of infra-red radiation reaching the operator's eyes and so reduce potential discomfort. The temperature of the stage was raised by using an electrical heating element, and the point at which the crystals just started to melt was noted. The routine was repeated using a fresh sample of hesperidin and a slower rate of heating. Again, the point at which the crystals started to melt and the range over which they melted was noted and recorded as the melting point range.

(b) Ultra Violet Absorption Spectroscopy

A sample of the recrystallised hesperidin was dissolved in methanol and its ultraviolet absorption determined using a Unicam SP800 spectrometer. The spectrum of the sample over the range 220 - 450 nm was recorded against a blank of methanol. The addition of small quantities of dilute sodium hydroxide or aluminium
trichloride to an alcoholic solution of flavanones produces characteristic shifts of their absorption spectra, so the effect of adding a few drops of 0.1N sodium hydroxide or 10 mg of aluminium trichloride to the methanolic solution of hesperidin was noted.

(c) Quantitative Estimation of Hesperidin

Initially two methods of quantitative evaluation were tried, these were an ultraviolet absorption method, and a colorimetric method attributable to Davis (1947). However, the Davis method (detailed in Appendix 5) was abandoned since it was found to be unreliable with low levels of hesperidin and also necessitated the use of an alkaline preparation which, for reasons to be presented in the discussion section, was considered undesirable.

(d) Ultraviolet Absorption Method

The procedure adopted was a modification of the method described by Hendrickson, Kesterton and Edwards (1959). The modifications were that both an alcoholic and aqueous solution of hesperidin were used, whereas the original only used an alcoholic solution. Also the original method required an alcoholic solution of hesperidin to which had been added some sodium hydroxide, and for reasons which will be considered in the discussion section of this thesis, this was considered inappropriate for this investigation.

Known weights of the recrystallised hesperidin were dissolved in either methanol (50 ml) or distilled water (50 ml). To these solutions were added 0.4 ml of 10% copper sulphate solution, and the resultant solutions were allowed to stand for 30 mins at room temperature. The U.V. absorption spectrum of these solutions were determined against the appropriate blank, i.e.: methanolic copper
sulphate solution or aqueous copper sulphate solution. The height of the absorption spectrum at 290 and 300 mp was noted. The difference in the absorbance at 290 mp minus that recorded at 300 mp is taken as a measure of hesperidin content. Thus, it was possible to prepare standard calibration curves of known amounts of hesperidin in either methanol or water versus the difference in absorption at 290 - 300 mp. The justification for using this approach and typical calibration curve will be presented in the results section.

4. The Identification of Crystalline Material from Orange Juices, Bases and Products

(a) Isolation

A portion of the various samples were diluted, where appropriate, with distilled water to give a solution of 11° Brix. This solution was then centrifuged at 360 x g for 10 mins and the supernatant or "cloud" retained. The cloud was then centrifuged at 27,000 x g for 1 hr to yield a sediment and clear supernatant. The sediment was resuspended in distilled water and the centrifugation at 27,000 x g repeated. The examination of the sediment using light microscopy indicated that it was rich in fine crystals.

(b) Melting Point determination

The isolated crystals were subjected to a melting point determination, as previously described, using the light microscope equipped with a heating stage.

(c) Ultraviolet Absorption Spectroscopy

A methanolic solution of the isolated crystalline material was
subjected to ultraviolet absorption spectroscopy as previously described. Additionally, the test solution was made alkaline with sodium hydroxide, or some aluminium trichloride was added, and the resultant spectrum noted.

5. Quantitative Estimation of Hesperidin in Juices and Bases

Samples of the juices and bases were again diluted to 11° Brix with distilled water and centrifuged at 360 x g for 10 mins. To 50 ml of the supernatant was added 0.4 ml of 10% copper sulphate solution and this mixture was allowed to stand for 30 mins. A portion of this solution was diluted 1 part to 20 parts with either methanol or distilled water. The ultraviolet spectrum was recorded as previously described, and the height of the spectrum at 290 and 300 nm noted. The difference in curve height (290-300) was taken as a measure of hesperidin content by comparison with the standard calibration curve.

6. Determination of Refractive Index of Hesperidin Crystals

The refractive index of the recrystallised hesperidin was determined using the Beeke line method (Shillaber, 1959). The crystals were placed on a microscope slide, and then immersed in a small volume of solution of known refractive index. The crystals were brought into focus, and then the direction of movement of the Beeke line, which is observed when the focus of the microscope is altered, was noted. When the microscope tube is lowered the line moves towards the material of lower refractive index. The solutions were changed until two solutions had been obtained where one solution was of lower refractive index than hesperidin, whilst the other was of greater index. This effectively brackets the refractive index.
of the hesperidin crystals. Admixes of these two solutions were then prepared until neither the specimen or Becke line were apparent, this point was taken as being the refractive index of the hesperidin. The actual refractive index of the solution was determined using an Abbe refractometer.

7. Solubility of Hesperidin in Synthetic Juice

In order to determine the solubility of hesperidin in orange juice, a synthetic juice was prepared by dissolving sucrose, citric acid and ascorbic acid in water. The proportions were as follows:

- Sucrose .......... 110g.
- Citric acid .... 15 g.
- Ascorbic acid .. 500mg.
- Make up to 1000g with water.

A sample of hesperidin (50 mg) was weighed out and the volume of synthetic juice required to dissolve this weight of hesperidin was determined at 20°C and 70°C.
FORMULATION OF A PRODUCT

Recipe for the preparation of a squash type of product which legislation dictates must contain at least 25% of single strength juice.

66° Brix juice .......... 104 ml.
Sucrose .................... 972.5 g.
10% Saccharin ............. 5 ml.
Citric acid ................. 16 g.
Sodium benzoate .......... 1.6 g.

Make up to 2.6 litres with water.
THE DETERMINATION OF THE OPTIMUM FORCE FOR CENTRIFUGING SAMPLES

A sample of base was diluted with distilled water to give a solution of 11° Brix. Aliquots (10 ml) of this solution were then spun at steps of 100xg from 100 - 1000xg for 10 minutes. The sediment and cloudy supernatant were then separated, and a small volume (1 ml) mixed with an equivalent volume of 2% phosphotungstic acid pH 5.5. A drop of this suspension was then placed on a collodion covered copper electron microscope grid, and allowed to dry.

The examination of the grids in the electron microscope indicated that both the sediment and cloudy supernatant contained crystals at all levels of centrifugation.

It was therefore concluded that no single force between 100 and 1000 x g offered the prospect of obtaining a fraction containing virtually all of the crystalline material. Therefore the force of 360 x g used by earlier workers was adopted for the routine studies, since results obtained using this level of centrifugation could then be compared with results obtained by other workers.
PREPARATION OF COVERED ELECTRON MICROSCOPE GRIDS

The 100 mesh copper electron microscope grids were covered with a thin film of collodion using the following method.

A 10 cm petri plate was filled with distilled water, and then a stainless steel gauze was immersed in the water and covered with copper grids. A drop of 1% collodion in amyl acetate was then placed on the surface of the water, and a thin collodion film formed by gentle evaporation of the amyl acetate. The water was then carefully drained away from the petri plate using a pipette, so that the collodion film descended to cover the copper grids. The steel gauze with the copper grids now covered with a wet collodion film was then removed and dried in an oven at 60°C. The copper grids which were covered with a dry collodion film were then ready for use.
DETERMINATION OF CLOUD LEVEL USING NEPHELOMETRY

The sample under investigation was diluted, where appropriate, with distilled water to give a solution of 11° Brix and centrifuged at 360 x g for 10 mins. The supernatant was then placed in a nephelometer tube and the reading on the nephelometer scale noted. It was then found that if the solution was diluted further the nephelometer readings indicated that the solutions had become more turbid, although visually the sample had become less turbid. Although this anomaly was reproducible over a range of dilutions, it was considered that these results bore little relationship to a visual assessment of cloud level so this method was abandoned.
The method for hesperidin analysis introduced by Davis (1947) is based on a colorimetric reaction between hesperidin and an alkaline solution of diethylene glycol.

The test sample was prepared by centrifuging an 11° Brix solution at 360 x g for 10 mins. To 0.2 ml of the supernatant was added 10 ml of 90% aqueous diethylene glycol and 0.2 ml of 4N sodium hydroxide solution. The mixture was allowed to stand for 30 mins, and then the intensity of the colour of the solution determined using an Eel absorptiometer at 420 mμ. The blank was prepared using the same solution of sample and diethylene glycol but water was used instead of the sodium hydroxide solution. A standard calibration curve was obtained by plotting the absorption obtained from methanolic solutions containing known quantities of recrystallised hesperidin. However with low levels of hesperidin this assay method gave very erratic results and was therefore abandoned.
RESULTS

During the course of this work a number of different techniques have been used to study the citrus samples, and a general guide to the presentation of the results is given below:

Morphological Features

1. Light microscopy.
2. Electron microscopy.

Physical and Chemical Characteristics

1. Quantitative estimates of crystals.
2. Identification and estimation of hesperidin.
3. Characterisation of fresh orange juice.
4. Hesperidin content of lemon juice.

Morphological Features

1. **LIGHT MICROSCOPY**

General Structure of Orange Tissues

(a) *Flavedo*

The outermost layer of epidermal cells comprised very small cells which appeared polygonal in plan view and were covered in a waxy cuticle. In transverse section the cells immediately below the cuticle layer were somewhat larger and isodiametric (Plate 1). These cells were rich in chromoplasts, which imparted the characteristic colour to the flavedo, and frequently contained calcium oxalate crystals which were evident when the sections were viewed under polarised light (Plate 2). The crystals were identified by their shape, and the fact that when the sections were irrigated with
dilute sulphuric acid, the crystals were transformed to needle shaped gypsum crystals.

One of the most striking features of a transverse section of the flavedo were the large oil glands (500 - 1,000μm in diameter) which were situated just below the epidermal cells (Plate 3). Within these glands there was some evidence for internal structure, although no fine detail was discernible. Polarised light illumination revealed the birefringent nature of the cell walls of the cells surrounding the oil glands, but little or no birefringent material was observed within the glands (Plate 4).

(b) Albedo

The cells in the albedo were not only much larger than those of the flavedo, but also had much thicker cell walls (Plate 5). Furthermore the cells were separated by large air filled intercellular spaces, and numerous strands of vascular tissue with characteristic thickened cell walls were also present (Plate 6). Calcium oxalate and hesperidin crystals were frequently found in close proximity to the vascular strands, and the tissue, as well as the crystals were highly birefringent under polarised light (Plate 7).

(c) Segment membrane

The cells of the segment membrane were found to be very similar to those of the albedo, and were also found to contain numerous crystals, later shown to be calcium oxalate and hesperidin.

(d) Juice sac

The epidermal cells of the juice sac were elongated in the plane of the juice sac wall and had a thick cuticle. However, the interior cells of the sac appeared to be rounded and contained numerous chromo-
plasts as well as calcium oxalate and hesperidin crystals (Plate 8).

Structure of Juices and Bases

The main particles visible in juices and bases consisted of various fragments of the original cell walls, as well as chromoplasts, crystals and other cellular debris. In some instances the cell nuclei were visible and these were normally identified under phase contrast by their dark nucleolus. Furthermore the nuclei were frequently surrounded by chromoplasts (Plate 9), as well as numerous needle like hesperidin crystals which were often formed into star shaped spherulites (Plate 10).

In both the juices and the bases the structures lacked contrast and were consequently difficult to photograph on black and white film, but because of the presence of chromatophores more effective representation was achieved on colour film (Plate 11).

Structure of products

In general the particles observed in the various commercially produced products were identical to those observed in the juices and bases. Some of the products prepared from bases exhibited a defect, which has been described by members of the trade as white cap. The white cap was a fairly thick layer of white material which was found immediately above the layer of tissue fragments at the bottom of the pack, and was composed virtually entirely of needle shaped crystals (Plate 12).

2. ELECTRON MICROSCOPY

(a) Transmission electron microscopy of thin sections

(i) Natural orange tissue

The cells of the flavedo (Plate 13) were 8 – 10 μm in diameter,
and bounded by cell walls of 1 - 2µm thickness which were traversed by plasmodesmata. The cytoplasm was densely stained by uranyl acetate and lead citrate, and the cells immediately beneath the epidermis contained numerous small vacuoles. In addition a centrally placed nucleus was obvious in most cells (Plate 14), as were densely staining droplets which were presumed to be lipid because of their reaction with osmium tetroxide. These lipid droplets which were between 0.5 - 1.0µm in diameter, were not bounded by a discernible membrane. The cells also contained densely staining mitochondria, and the cristae within some of these appeared to be swollen and distorted.

The dominant feature of the cytoplasm of the layer of cells near the albedo was a large central vacuole or tonoplast which was surrounded by a thin parietal layer of cytoplasm (Plate 15). Nevertheless, the cytoplasm contained all the normal organelles, including a nucleus, mitochondria, lipid droplets as well as numerous small vesicles. The cell walls were thicker (up to 4µm) and exhibited more characteristic pit-field structures. Furthermore, small intercellular spaces occurred occasionally (Plate 15), and these cells were clearly transitional between typical flavedo and albedo cells.

The albedo cells were 20 - 50µm diameter and had extremely thick cell walls up to 20µm across which contained pit-fields which bore numerous plasmodesma (Plate 16). The cytoplasm was normally a thin layer compressed against the cell wall (Plate 17). The cells were separated by massive intercellular spaces 20 - 40µm in diameter (Plates 18 and 19). The combination of very thick cell walls and large intercellular spaces made it very difficult to obtain satisfactory thin sections, and virtually impossible to obtain sections of entire cells whose walls did not exhibit some compression or folding.
The segment membrane was also found to be a fairly difficult tissue to section. The cells of this tissue were 8 - 20\(\mu\)m in diameter and surrounded by relatively thick cell walls 2 - 6\(\mu\)m diameter (Plates 20 and 21). The cell walls frequently exhibited a marked fibrous pattern (Plate 22), and had an electron opaque precipitate associated with them. This precipitate was probably an artifact, appeared to be specific to the wall region, and was not generally observed on other parts of the section. The cytoplasm of these cells often appeared to be slightly plasmolysed, with the plasma membrane lying away from the cell wall. However, the various cytoplasmic organelles could be identified, although they were usually poorly preserved when compared with the equivalent organelles found in the other tissues of the orange.

The outermost cells of the juice sac were columnar and covered in a cuticle (Plate 23), whereas the lower cells were polygonal and 5 - 14\(\mu\)m in diameter. These cells contained tonoplasts of variable size, some occupying a major portion of the cytoplasm, whilst others appeared as small vesicles. The cells also contained nuclei, chromoplasts and densely staining droplets which were presumed to be lipid. The cells lying beneath the 5 or 6 outer layers tended to contain numerous vacuolar vesicles, lipid droplets and chromoplasts (Plate 24). The walls of the outer cells were 0.5 - 1.0\(\mu\)m thick, but those round the cells at the centre of the juice sac were very much thinner being 10 - 30nm in diameter (Plate 25). There was little evidence of a gradation in wall thickness between these two extremes, but by tracing the thin walls back to their branching point from the relatively thick walls (Plate 26), it was evident that they were of similar structure and function.

The cuticle layer on the outermost cells of the juice sac was
about 0.2 μm thick (Plate 27) and sometimes showed evidence for other cellular debris adhering to it (Plate 28). The cell walls contained fairly well defined pit-fields with plasmodesma traversing the wall. The cytoplasm of the cells from the first 5 - 8 layers of cells contained a large nucleus, with nucleolus, chromatin and well preserved nuclear membrane (Plate 29). Also present were vacuolar vesicles, mitochondria, lipid droplets and chromoplasts (Plate 29). The chromoplasts were organelles 3 - 4 μm long and 1 μm diameter, usually with an elongated or dumb-bell form and bounded by a membrane, they contained a granular or membranous interior with densely staining droplets frequently arranged in a circular array (Plate 30).

Within the central cells the walls were only 10 - 30 nm thick, there was no evidence of pit-fields or plasmodesma (Plate 31), and it was frequently difficult to discern an intact plasma membrane. However, in some cells there were numerous membrane bound vesicles (Plate 32). Occasionally, nuclei were observed within these cells, as were mitochondria, but they were generally poorly preserved indicating that some autolysis may have taken place. A striking feature in some areas were densely staining droplets 0.5 - 1.0 μm diameter, which were typically sited close to the cell wall or associated with aggregates of vesicles (Plate 33).

(ii) Sections of Single Strength Juice

The sections obtained from the microencapsulated single strength juice revealed a range of cell walls and cellular debris (Plate 34). The cell wall material consisted of two fairly distinct types. Relatively thick walls (0.5 - 1.5 μm thick) some of which exhibited a densely staining cuticle layer, and very much thinner walls, some of which were only 10 - 20 nanometers in width. Much of the
cellular debris was either vesicle like or granular material of less than 50 nm diameter. There was also evidence for distinct aggregates of vesicles and densely staining droplets many of which were within the range 0.5 - 2.0 µm diameter, although some were considerably larger (Plate 35). In addition to the vesicles associated with densely staining droplets, there were numerous arrays of vesicles, membranes and granular structures, some of which formed quite extensive structures (Plate 36).

(iii) Sections of Concentrated Juices

The appearance of a six fold concentrated juice (i.e.: 66° Brix) is illustrated on Plates 37 and 38. The dominant features were cell walls which varied from 0.02 - 1.0 µm in width, and other readily discernible structures were chromoplasts, densely staining droplets and numerous small vesicles. Also characteristic of these heat concentrated juices were aggregates of very small densely staining particles (Plate 39), never seen in fresh tissues nor observed to any great extent in single strength juice which had been "pasteurised". The particles were 10-30 nm in diameter, and were quite frequently associated with other vesicular material (Plate 40). They are presumed to have resulted from the heat treatment used for concentrating the juices.

Some of the cell walls observed in the concentrated juices exhibited the densely staining cuticle layer which is considered to be characteristic of the outer cells of the juice sac (Plate 41).

(iv) Sections of Bases

The examination of thin sections of the microencapsulated bases indicated that in addition to the types of structures seen in the concentrated juices numerous very thick wall structures were also
present (Plates 42 and 43). These walls were 6 - 10 µm thick and from their general appearance it was apparent that they were originally derived from flavedo, albedo and segment membranes. The very much thinner cell walls observed in the juices were also present, as were densely staining droplets, vesicles and the densely staining particles (Plate 44) noted in the heat concentrated juices.

(v) Sections of commercial products

In general the various products contained the same type of particles observed in the juices and bases, except for material in the neck rings which developed at the top of the packs.

The material obtained from the sediment of a commercial product comprised mainly of thick cell walls and general cellular debris (Plates 45 and 46). In order to obtain a sufficient concentration of the cloud material for microencapsulation, the cloud from the products was collected by centrifugation at 28,000 x g. The sediment from the centrifugate was then resuspended in a small volume of the supernatant, and then this "concentrated cloud" was microencapsulated as described above. Thin sections of this material revealed that it was composed of thin cell walls, densely staining droplets, vesicles and fine granular material (Plates 47 and 48). It was difficult to judge whether the concentration of the different particles were the same in all the samples, since the method of specimen preparation, i.e.: microencapsulation made it impossible to guarantee that each microcapsule received an identical number of particles, or that the distribution within the microcapsules would be similar. For these considerations it would have been meaningless to have attempted to obtain any quantitative data on particle numbers from thin sections of microencapsulated materials.

The structures observed in the neck rings of products formulated
from a base prepared by blending together juice and peel tissues, were quite different from those observed from products formulated from bases prepared by comminuting whole oranges.

The main structural feature of the neck ring from the juice and peel type of product was a dense population of roughly spherical particles with diameters ranging from 0.5 - 5.0 \( \mu \)m (Plates 49 and 50). The majority of the particles were bounded by a fine granular material and contained within their centres relatively amorphous, granular materials and densely staining small droplets of approximately 0.1 \( \mu \)m diameter. Many of these densely staining droplets were arranged close to the periphery of the larger particles (Plate 50). Some of the particles contained complex arrangements of granular materials, membranes and structures with a geometrical crystalline symmetry (Plate 51).

The neck rings from the products prepared from comminuted whole oranges contained predominantly empty droplets bounded by a densely staining membrane (Plates 52 and 53). The droplets ranged from 0.1 - 2.0 \( \mu \)m in diameter, and the bounding membrane was about 25 - 60 nm thick. Other materials that were observed included densely staining very fine granular materials, as well as some free membrane structures and short lengths of relatively thin cell walls.

(b) Transmission electron microscopy and freeze etching

(i) Single strength juices without cryoprotectant

There was a characteristic reticular matrix appearance of these specimens which was probably attributable to the formation of ice crystals. Embedded within this matrix were cell walls with an apparent width of about 0.1 \( \mu \)m, as well as numerous droplets (Plate 54). At a higher magnification needle like crystals were also revealed (Plate 55).
(ii) **Single strength juices with a cryoprotectant**

In a glycerinated juice the icy matrix had been reduced and many aggregates of small droplets and some crystals were apparent (Plate 56), the droplets varying from 40 - 250 nm in diameter.

(iii) **Concentrated juices and bases without cryoprotectant**

The incorporation of a cryoprotectant was unnecessary for adequate preparation of these samples, because the ice formation was reduced by the high levels of soluble solids such as sugar. The predominant structures in a representative base were aggregates of small droplets (Plate 57), which like those present in the juice samples were 40 - 250 nm diameter. Furthermore, the size of the aggregates were similar being 0.5 - 1.5 \( \mu \)m diameter. However, some much larger aggregates were also observed, which were frequently ellipsoidal rather than spherical in form and thus 3.0 x 1.5 \( \mu \)m across. Some of the aggregates appeared to be composed of mainly droplets (Plate 58) with only limited amounts of crystalline material, whilst other aggregates contained numerous crystalline bodies (Plate 59). It was difficult to assign a precise size to the crystals, because only rarely was the plane of the fracture aligned with a plane of crystal symmetry and a whole crystal exposed. However, the examination of a number of freeze etch replicas indicated that the crystals had fracture face dimensions of the order of 0.1 \( \mu \)m. It was interesting to observe that none of the aggregates of droplets and crystals appeared to be bounded by a limiting membrane. On some replicas quite extensive regions of crystalline material were observed (Plate 60), and the appearance of some of these crystal aggregates was indicative of star shaped spherulites as observed by light microscopy. In certain fracture planes the crystals also showed
evidence for layer growth (Plate 60) with the growth steps being about 3 nm.

In addition to droplets and crystals, some of the aggregates in both the juices and bases contained material that had either a particulate or smooth appearance (Plates 61 and 62). The dimensions of the particulate material was of the order of 10 – 30 nm and was probably analogous to the densely staining particulate material observed in the thin sections of heat concentrated juices and bases. Cell wall fragments were not observed in the cryoprotected juices or the concentrated juices and bases.

(iv) Neck rings from products prepared from bases

Material obtained from the neck rings from bases prepared from juice plus peel contained large droplets 0.5 – 5.0 μm diameter (Plate 63). They were not bounded by a distinct membrane, but tended to have a reticular surface where this was in contact with the aqueous phase. Furthermore the internal structure of the droplets was not homogenous, but contained some distinct smaller droplets (Plate 64).

The neck ring droplets present in products made from the base prepared from a comminuted whole orange appeared to be bounded by a distinct membrane 30 – 40 nm thick (Plate 65). The droplets were about 0.1 – 2.0 μm across, and the interior was composed of relatively homogenous material.

(c) Transmission electron microscopy and negative staining

The initial method of specimen preparation involved centrifuging a single strength juice to produce a cloud fraction, and then mixing a proportion of the cloud with negative stain and drying down onto a collodion covered grid. Such specimen preparations contained apparently circular or spherical electron opaque particles 0.15 – 0.3 μm diameter (Plate 66). However, it was noticed that these
types of structures appeared to be formed during the prolonged irradiation of the specimen grid with the electron beam. Furthermore, when these spherical structures were forming on the grid, needle shaped crystals became apparent amongst the negative stain (Plate 67). It was concluded that some material from the juice which had been deposited onto the grid was being volatilised under the conditions of vacuum and irradiation with the electron beam with the result that spurious artifacts were produced. The sediment from orange juice subject to centrifugation at 360 x g did not exhibit this artifact, and was composed of predominantly crystals and cell wall materials (Plate 68). The densely staining material with which the crystals were usually associated was fibrillar in nature (Plate 69).

An alternative preparative technique was to collect the cloud at 30,000 x g. The deposit was washed twice by resuspending it in distilled water followed by centrifugation. Negatively stained preparation of the washed deposit did not alter under the electron beam and typical results are shown on Plate 70, revealing numerous needle shaped crystals. These were mainly small (0.6 - 1.0 μm long by 50 - 60 nm wide) but large crystals were also present (2 μm long by 200 nm wide).

Using the cloud washing procedure outlined above the range of negative stains listed in the Materials and Methods Section were evaluated to see which stain gave the best results in terms of reproducibility and contrast, bearing in mind that the pH of the juices and bases were between pH 4.5 - 5.5.

An aqueous solution of phosphotungstic acid at pH 1.0 did not act as a negative stain but gave a black precipitate all over the grid, but at pH 5.0 and 7.0 the stain gave adequate and comparable results. Uranyl acetate and ammonium molybdate (both of pH 6.0)
did not produce as much contrast as the phosphotungstic stains. Thus phosphotungstic acid at pH 5.0 was selected as the standard throughout this work because at that pH the stain was nearest to the pH of the orange samples. The cloud derived from a base which had been washed and resuspended was similar to an equivalent preparation obtained from the juice (Plate 71). The main features were crystals both small and large (15 nm wide 0.3 μm long; 60 nm wide and 0.6 μm long). In addition small circular or spherical particles were present about 15 - 60 nm in diameter, as well as particulate material with a size range of 20 - 200 nm, and membrane bound particles which had the appearance of flattened vesicles (Plate 72). Occasionally structures were seen which resembled cellular organelles, an example of which is illustrated on Plate 73 and which probably represents golgi bodies.

The examination of the cloud from a negatively stained lemon juice also revealed numerous needle shaped crystals (Plate 74). These crystals were of similar appearance to those observed in orange juice, except that some of the crystals were very long (up to 10 μm long and 0.3 μm wide).

(d) Scanning electron microscopy

The examination of the cloud isolated from a juice by centrifugation (30,000 x g) revealed a mass of needle like crystals (Plate 75), which were shown by Xray spectroscopy to be composed of carbon, hydrogen, oxygen and calcium.

The inner surface of the juice sacs were also examined by scanning electron microscopy. Fresh juice sacs were emptied by a syringe and slit open with a scalpel. The membranous tissue was then freeze dried onto specimen stubs with the inner surface uppermost. After it had been coated in the normal manner with a heavy
metal, examination revealed a slightly wrinkled surface, free of other structural features and without any crystalline deposits (Plate 76).

Physical and Chemical Characteristics

1. Size of the crystals in orange samples

The measurement of the sizes of the crystals observed in the negative stain preparations of the various orange samples, revealed that the width dimensions were all less than $0.25 \mu m$, whilst the lengths varied from 0.6 up to $4.0 \mu m$ (Table 4). With the exception of the base Israeli 30$^0$ Brix (sample 2), the crystals in the bases were smaller than those observed in either the juices or products, which were generally of a similar size.

2. Concentration of crystals in orange samples

The number of crystals in 10 fields of view of a negatively stained preparation were counted, and the mean value was related to the number of latex spheres also noted in the same area. Thus, a correction factor could be applied when necessary to ensure that equivalent volumes of material had been dried down onto the grid. In practice it was found that with care equivalent volumes of negatively stained samples could be applied to the grid and correction factors were unnecessary. The number of crystals found in the various samples are listed in Table 5. The range of the concentration of crystals in any given type of sample i.e.: juice, base or product was found to be quite wide (e.g.: juices 7 - 88). The greatest numbers of crystals were in the bases, and the lowest numbers in the products.
TABLE 4 Dimensions of crystals in orange samples
(Mean of 20 measurements)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Width $\mu$m</th>
<th>Length $\mu$m</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Orange Juice</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Israeli 11° Brix</td>
<td>0.19 ± 0.04</td>
<td>3.5 ± 0.7</td>
</tr>
<tr>
<td>Israeli 44° Brix</td>
<td>0.23 ± 0.07</td>
<td>2.1 ± 0.7</td>
</tr>
<tr>
<td>Israeli 66° Brix</td>
<td>0.12 ± 0.04</td>
<td>1.6 ± 0.8</td>
</tr>
<tr>
<td>(Sample 1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Israeli 66° Brix</td>
<td>0.14 ± 0.02</td>
<td>2.0 ± 0.7</td>
</tr>
<tr>
<td>(Sample 2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spanish 55° Brix</td>
<td>0.12 ± 0.03</td>
<td>1.05 ± 0.7</td>
</tr>
<tr>
<td>South African 66° Brix</td>
<td>0.11 ± 0.01</td>
<td>1.15 ± 0.26</td>
</tr>
<tr>
<td><strong>Orange Bases</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Israeli 30° Brix</td>
<td>0.06 ± 0.01</td>
<td>0.6 ± 0.14</td>
</tr>
<tr>
<td>(Sample 1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Israeli 30° Brix</td>
<td>0.21 ± 0.13</td>
<td>4.0 ± 2.2</td>
</tr>
<tr>
<td>(Sample 2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Israeli 30° Brix</td>
<td>0.09 ± 0.05</td>
<td>0.72 ± 0.2</td>
</tr>
<tr>
<td>(Sample 3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spanish 200%</td>
<td>0.07 ± 0.02</td>
<td>0.69 ± 0.14</td>
</tr>
<tr>
<td>Spanish 30° Brix</td>
<td>0.09 ± 0.02</td>
<td>0.89 ± 0.34</td>
</tr>
<tr>
<td><strong>Orange Products</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Israeli High Cloud(HC)</td>
<td>0.12 ± 0.01</td>
<td>1.9 ± 0.9</td>
</tr>
<tr>
<td>Israeli Low Cloud(LC)</td>
<td>0.11 ± 0.04</td>
<td>1.8 ± 0.9</td>
</tr>
<tr>
<td>Israeli HC/LC blend</td>
<td>0.12 ± 0.02</td>
<td>2.6 ± 0.75</td>
</tr>
<tr>
<td>Italian Low cloud(LC)</td>
<td>0.11 ± 0.02</td>
<td>28 ± 15</td>
</tr>
<tr>
<td>Spanish Low cloud(LC)</td>
<td>0.14 ± 0.02</td>
<td>2.3 ± 0.5</td>
</tr>
<tr>
<td>Israeli + Spanish</td>
<td>0.15 ± 0.05</td>
<td>2.9 ± 1.4</td>
</tr>
</tbody>
</table>
### TABLE 5  
**Number of Crystals present in the Sample**  
Diluted to the equivalent of 11° Brix.

<table>
<thead>
<tr>
<th>Type</th>
<th>No. of Crystals per Latex Sphere Basis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Orange Juice</strong></td>
<td></td>
</tr>
<tr>
<td>Israeli 11° Brix</td>
<td>12</td>
</tr>
<tr>
<td>Israeli 44° Brix</td>
<td>88</td>
</tr>
<tr>
<td>Israeli 66° Brix</td>
<td>7</td>
</tr>
<tr>
<td>(Sample 1)</td>
<td></td>
</tr>
<tr>
<td>Israeli 66° Brix</td>
<td>25</td>
</tr>
<tr>
<td>(Sample 2)</td>
<td></td>
</tr>
<tr>
<td>Spanish 55° Brix</td>
<td>70</td>
</tr>
<tr>
<td>South African 66° Brix</td>
<td>20</td>
</tr>
<tr>
<td><strong>Orange Bases</strong></td>
<td></td>
</tr>
<tr>
<td>Israeli 30° Brix</td>
<td>135</td>
</tr>
<tr>
<td>(Sample 1)</td>
<td></td>
</tr>
<tr>
<td>Israeli 30° Brix</td>
<td>48</td>
</tr>
<tr>
<td>(Sample 2)</td>
<td></td>
</tr>
<tr>
<td>Israeli 30° Brix</td>
<td>90</td>
</tr>
<tr>
<td>(Sample 3)</td>
<td></td>
</tr>
<tr>
<td>Spanish 200%</td>
<td>96</td>
</tr>
<tr>
<td>Spanish 30° Brix</td>
<td>22</td>
</tr>
<tr>
<td><strong>Orange Products</strong></td>
<td></td>
</tr>
<tr>
<td>Israeli High cloud (HC)</td>
<td>45</td>
</tr>
<tr>
<td>Israeli Low cloud (LC)</td>
<td>9</td>
</tr>
<tr>
<td>Israeli HC/LC blend</td>
<td>24</td>
</tr>
<tr>
<td>Italian Low cloud (LC)</td>
<td>6</td>
</tr>
<tr>
<td>Spanish Low cloud (LC)</td>
<td>9</td>
</tr>
<tr>
<td>Israeli + Spanish</td>
<td>30</td>
</tr>
</tbody>
</table>
3. Measurement of Cloud Level

The Eel absorptiometer was calibrated against suspensions of bentonite, and the calibration curve at 660 m\(\mu\) is shown in Fig. 5. On the basis of this standard it was possible to determine the relative cloud values of the various orange samples, and relate these observations to those of other workers who had adopted the same standard procedure. The cloud levels found in the orange juices, bases and products are listed in Table 6, and reveal a wide range of cloudiness. The absorptiometer readings agree in general with the commercial evaluation of the sample cloud level. But the instrumental values had the advantage of providing a much more sensitive assay, and gave values for samples which were rated as "normal" although just noticeably different.

The technique was equally suitable for the evaluation of the cloud levels on the lemon juices (Table 7). Even with this somewhat limited range of lemon juices there was again good agreement between the instrumental and subjective evaluation of cloud level.

The microscopical examination of the various orange samples indicated that one of the most strikingly different features between them were the number of crystals present in the cloud, and a plot of cloud level against crystal number, for the juices, bases and products, revealed a linear relationship (Fig. 6, 7 and 8), which clearly indicated that the number of crystals present in a juice might well be a significant factor in determining cloud level. However since the plot did not pass through zero, but was always above the axis, it was clear that the concentration of crystals was not the sole factor involved in cloudiness.
Fig. 5. Calibration curve of Eel Absorptiometer using bentonite suspensions at 660 m\( \mu \) and 1 cm cells.
<table>
<thead>
<tr>
<th>Type</th>
<th>Dilution Factor of Cloud after Centrifugation at 360 x g</th>
<th>Absorbance Reading at 660 m/μ</th>
<th>Commercial Evaluation of Cloud</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Orange Juice</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Israeli 11° Brix</td>
<td>3</td>
<td>28.5</td>
<td>Normal</td>
</tr>
<tr>
<td>Israeli 44° Brix</td>
<td>3</td>
<td>97</td>
<td>High Cloud</td>
</tr>
<tr>
<td>Israeli 66° Brix</td>
<td>3</td>
<td>26.5</td>
<td>Normal</td>
</tr>
<tr>
<td>(Sample 1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Israeli 66° Brix</td>
<td>3</td>
<td>43</td>
<td>Normal</td>
</tr>
<tr>
<td>(Sample 2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spanish 55° Brix</td>
<td>3</td>
<td>88</td>
<td>Good Cloud</td>
</tr>
<tr>
<td>South African 66° Brix</td>
<td>3</td>
<td>35</td>
<td>Normal</td>
</tr>
<tr>
<td><strong>Orange Bases</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Israeli 30° Brix</td>
<td>4</td>
<td>76</td>
<td>High Cloud</td>
</tr>
<tr>
<td>(Sample 1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Israeli 30° Brix</td>
<td>4</td>
<td>39</td>
<td>Normal</td>
</tr>
<tr>
<td>(Sample 2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Israeli 30° Brix</td>
<td>4</td>
<td>58</td>
<td>Good</td>
</tr>
<tr>
<td>(Sample 3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spanish 200%</td>
<td>4</td>
<td>56</td>
<td>Good</td>
</tr>
<tr>
<td>Spanish 30° Brix</td>
<td>4</td>
<td>28</td>
<td>Low Cloud</td>
</tr>
<tr>
<td><strong>Orange Products</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Israeli High Cloud</td>
<td>3</td>
<td>28</td>
<td>High Cloud</td>
</tr>
<tr>
<td>Israeli Low Cloud</td>
<td>3</td>
<td>14</td>
<td>Low Cloud</td>
</tr>
<tr>
<td>Israeli HC/LC blend</td>
<td>3</td>
<td>17</td>
<td>Good Cloud</td>
</tr>
<tr>
<td>Italian Low cloud</td>
<td>3</td>
<td>12</td>
<td>Low Cloud</td>
</tr>
<tr>
<td>Spanish Low cloud</td>
<td>3</td>
<td>14</td>
<td>Low Cloud</td>
</tr>
<tr>
<td>Israeli/Spanish</td>
<td>3</td>
<td>22</td>
<td>Good Cloud</td>
</tr>
</tbody>
</table>
TABLE 7 Cloud level of lemon juices compared to commercial evaluation

<table>
<thead>
<tr>
<th>Type</th>
<th>Dilution Factor of Cloud after centrifugation at 360 x g</th>
<th>Absorbance Reading at 660 m(\mu)</th>
<th>Commercial Evaluation of Cloud</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sicilian 400 HC 1973 Season</td>
<td>5</td>
<td>54</td>
<td>High Cloud</td>
</tr>
<tr>
<td>Sicilian 400</td>
<td>5</td>
<td>27</td>
<td>Normal</td>
</tr>
<tr>
<td>New Sicilian 400 - HC</td>
<td>5</td>
<td>95</td>
<td>Very High Cloud</td>
</tr>
<tr>
<td>Sicilian 400 HC 1974 Season</td>
<td>5</td>
<td>60</td>
<td>High Cloud</td>
</tr>
</tbody>
</table>
Fig. 6. The relationship between cloud absorption versus number of crystals in orange juice.
Fig. 7. The relationship between cloud absorption versus number of crystals in an orange base.
Fig. 8. The relationship between cloud absorption versus number of crystals in an orange product.
4. Chemical characterisation of hesperidin and cloud crystals

(a) Ultraviolet spectroscopy

A sample of the recrystallised commercial hesperidin was dissolved in methanol and the ultraviolet absorption spectrum was determined. The spectrum is illustrated in Fig. 9, where it can be seen that the principle absorption occurs at 285 mμ, with a small peak at 327 mμ. The spectral shift of the alcoholic solution of hesperidin in the presence of either sodium hydroxide or aluminium trichloride is shown in Table 8.

TABLE 8 Absorption maxima of hesperidin solutions

<table>
<thead>
<tr>
<th>Solution of hesperidin in</th>
<th>Absorption maxima mμ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>284</td>
</tr>
<tr>
<td>Methanol plus sodium hydroxide</td>
<td>287</td>
</tr>
<tr>
<td>Methanol plus aluminium trichloride</td>
<td>308</td>
</tr>
</tbody>
</table>

It can be seen that the addition of sodium hydroxide produces only a small shift in the maxima from 285 to 287 mμ, whilst the aluminium trichloride shifts the maxima to 308 mμ.

The absorption spectrum of the crystalline material isolated from orange juice cloud by methanol is shown in Fig. 10, and it can be seen that the main peak is at 284 mμ, with a much smaller peak at 327 mμ. The spectral shifts when this methanol extract was treated with sodium hydroxide or aluminium trichloride are listed in Table 9.
Fig. 9. Ultraviolet absorption spectrum of hesperidin in methanol.
Fig. 10. Ultraviolet absorption spectrum of crystalline material isolated from orange juice in methanol.
TABLE 9  Absorption maxima of methanolic extract of orange juice cloud

<table>
<thead>
<tr>
<th>Solution</th>
<th>Absorption maxima m(\mu)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol alone</td>
<td>284</td>
</tr>
<tr>
<td>Methanol plus sodium hydroxide</td>
<td>287</td>
</tr>
<tr>
<td>Methanol plus aluminium trichloride</td>
<td>308</td>
</tr>
</tbody>
</table>

The agreement between the data of Tables 8 and 9 indicated that the crystalline material in the orange juice cloud was probably hesperidin. Similar analyses on the crystals from the cloud of bases and products indicated that they were also hesperidin.

(b) Melting Point

Further confirmation that the crystals were hesperidin was obtained from the melting point determinations. The melting point range of the recrystallised commercial hesperidin was 261 - 263°C and the range for the crystalline material extracted from juice was found to be identical.

5. Quantitative estimation of hesperidin in juices, bases and products

A calibration curve for the Hendrickson et al. (1959) method of determining hesperidin is shown in Fig. 11, and was found to give a linear relationship over the range 0 - 400 mg of hesperidin in 100 ml. of methanol. The calibration curve was obtained by dissolving known amounts of recrystallised hesperidin in methanol and noting the absorbance difference at 300 and 290 m\(\lambda\), this figure
Fig. 11. Calibration curve of hesperidin in methanol (dilution 1:20) using the modified Hendrickson method.
was then plotted against the known hesperidin content of the sample. If a spectrum of a sample was obtained which had an equal absorbance at 300 and 290 nm then this would indicate that no hesperidin was present compared with the methanol blank in the reference cell. This contention was confirmed, and the validity of the assay checked by determining the hesperidin content of a sample, and then adding this quantity of hesperidin to the methanol in the reference cell in the spectrophotometer, the absorbance at 300 and 290 nm then became equal.

One of the notable properties of hesperidin is its low solubility in water (1 g in 50 litres in cold water). However, it is readily soluble in alcohols, sodium hydroxide and pyridine (Merck Index, 1965). Therefore, a methanol extract of orange juice assayed as described above gave a measure of the total of both soluble and crystalline hesperidin. An aqueous extract of the same orange juice gave a much lower value for hesperidin content, since this was measuring only the soluble hesperidin. Thus by subtracting the value of an aqueous extract from a methanol extract the value for the insoluble (or crystalline) fraction of hesperidin was obtained. The results obtained from such determinations on the various samples of juices and bases are recorded in Table 10. Unfortunately, the products examined during the initial stages of the project had nearly all formed a substantial "white cap" at the bottom of the pack as a result of prolonged storage. Analysis of this material indicated that it was predominantly hesperidin, therefore an assay for hesperidin in the stored products bore little relationship to the original hesperidin content of the fresh products, so that the products have not been included in Table 10.

The results indicate that there was little relationship between the cloud level and total hesperidin (methanol extract) for the juices
<table>
<thead>
<tr>
<th>Type</th>
<th>Cloud reading</th>
<th>Total hesperidin content mg% Methanol Extract</th>
<th>Hesperidin content of aqueous extract mg%</th>
<th>Crystalline hesperidin content mg% Methanol-aqueous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orange Juice</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Israeli 11° Brix</td>
<td>28</td>
<td>120</td>
<td>110</td>
<td>10</td>
</tr>
<tr>
<td>Israeli 44° Brix</td>
<td>97</td>
<td>250</td>
<td>146</td>
<td>104</td>
</tr>
<tr>
<td>Israeli 66° Brix</td>
<td>26</td>
<td>70</td>
<td>60</td>
<td>10</td>
</tr>
<tr>
<td>(Sample 1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Israeli 66° Brix</td>
<td>43</td>
<td>134</td>
<td>100</td>
<td>34</td>
</tr>
<tr>
<td>(Sample 2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spanish 55° Brix</td>
<td>88</td>
<td>274</td>
<td>180</td>
<td>94</td>
</tr>
<tr>
<td>South African</td>
<td>35</td>
<td>168</td>
<td>145</td>
<td>23</td>
</tr>
<tr>
<td>66° Brix</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Orange Bases</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Israeli 30° Brix</td>
<td>76</td>
<td>220</td>
<td>120</td>
<td>100</td>
</tr>
<tr>
<td>(Sample 1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Israeli 30° Brix</td>
<td>39</td>
<td>160</td>
<td>120</td>
<td>40</td>
</tr>
<tr>
<td>(Sample 2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Israeli 30° Brix</td>
<td>58</td>
<td>240</td>
<td>160</td>
<td>80</td>
</tr>
<tr>
<td>(Sample 3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spanish 200%</td>
<td>56</td>
<td>240</td>
<td>168</td>
<td>72</td>
</tr>
<tr>
<td>Spanish 30° Brix</td>
<td>28</td>
<td>184</td>
<td>160</td>
<td>24</td>
</tr>
</tbody>
</table>
and bases. However, the linear relationship between cloud level and crystalline hesperidin content is illustrated in Figs. 12 and 13.

6. Refractive Index of hesperidin

The refractive index of the recrystallised hesperidin and some of the crystals isolated from cloud was found to be 1.595.

7. Solubility of hesperidin in synthetic orange juice

The solubility of the recrystallised hesperidin in the synthetic juice was found to be 1 g in 50 litres at room temperature and 1 g in 5 litres at 70°C.

8. Characterisation of fresh orange juice

(a) Effect of aging the juice

Oranges were reamed by hand, the juice was strained and then the cloud fraction was isolated by centrifugation at 360 xg. The cloud level was read on the absorptiometer and the hesperidin content assayed in a methanolic and aqueous extract of the cloud. A portion of the cloud was also negatively stained and a crystal count determined. The remainder of the sample was aged for 48 hours at 4°C, and then the cloud level, hesperidin assay and crystal count repeated. This experiment was repeated on 3 samples of juice and the results are contained in Table 11.

It can be seen that in the fresh juice there are relatively few crystals present, and furthermore the values for the hesperidin content in the aqueous and methanol extracts are very similar. However, in the aged juice there is an increase in the number of crystals present, and the hesperidin assay shows a marked decrease in the hesperidin content of the aqueous extract, although the value for the methanol extract stays approximately constant. This may be
Fig. 12. The relationship between cloud absorption versus hesperidin content for orange juices.
Fig. 13. The relationship between cloud absorption versus hesperidin content for orange bases.


<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>FRESH</th>
<th>48 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1) Cloud reading @ 660 μm</td>
<td>42</td>
<td>66</td>
</tr>
<tr>
<td>(2) Hesperidin content of methanol extract</td>
<td>165 mg%</td>
<td>170 mg%</td>
</tr>
<tr>
<td>(3) Hesperidin content of aqueous extract</td>
<td>160 mg%</td>
<td>110 mg%</td>
</tr>
<tr>
<td>(4) Crystalline hesperidin content (2-3)</td>
<td>5 mg%</td>
<td>60 mg%</td>
</tr>
<tr>
<td>(5) Crystal count</td>
<td>1</td>
<td>18</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1) Cloud reading @ 660 μm</td>
<td>50</td>
<td>68</td>
</tr>
<tr>
<td>(2) Hesperidin content of methanol extract</td>
<td>115 mg%</td>
<td>120 mg%</td>
</tr>
<tr>
<td>(3) Hesperidin content of aqueous extract</td>
<td>112 mg%</td>
<td>80 mg%</td>
</tr>
<tr>
<td>(4) Crystalline hesperidin content (2-3)</td>
<td>3 mg%</td>
<td>40 mg%</td>
</tr>
<tr>
<td>(5) Crystal count</td>
<td>2</td>
<td>12</td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1) Cloud reading @ 660 μm</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>(2) Hesperidin content of methanol extract</td>
<td>140 mg%</td>
<td>142 mg%</td>
</tr>
<tr>
<td>(3) Hesperidin content of aqueous extract</td>
<td>135 mg%</td>
<td>100 mg%</td>
</tr>
<tr>
<td>(4) Crystalline hesperidin content (2-3)</td>
<td>5 mg%</td>
<td>42 mg%</td>
</tr>
<tr>
<td>(5) Crystal count</td>
<td>1</td>
<td>12</td>
</tr>
</tbody>
</table>
interpreted as an increase of the crystalline phase of hesperidin on aging, and in each case is also accompanied by a concomitant rise in the cloud level. After 72 hrs there was a gradual clarification of the cloud in the samples, and this was probably due to the action of pectin methyl esterase. The experiment was repeated except that a portion of the freshly expressed juice was "pasteurised" by heating to 85°C for 5 minutes using a water bath. The results are contained in Table 12.

TABLE 12 The effect of aging "pasteurised" orange juice for 48 hrs at +4°C

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>Fresh</th>
<th>PASTEURISED 85°C for 5 min</th>
<th>48 hrs.</th>
<th>PASTEURISED 85°C for 5 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UNHEATED</td>
<td></td>
<td></td>
<td>UNHEATED</td>
</tr>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1) Cloud reading @ 660mp</td>
<td>43</td>
<td>43</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>(2) Hesperidin content of methanol extract</td>
<td>151 mg%</td>
<td>152 mg%</td>
<td>153 mg%</td>
<td>153 mg%</td>
</tr>
<tr>
<td>(3) Hesperidin content of aqueous extract</td>
<td>147 mg%</td>
<td>151 mg%</td>
<td>116 mg%</td>
<td>115 mg%</td>
</tr>
<tr>
<td>(4) Crystalline hesperidin content (2-3)</td>
<td>4 mg%</td>
<td>1 mg%</td>
<td>37 mg%</td>
<td>38 mg%</td>
</tr>
<tr>
<td>(5) Crystal Count</td>
<td>2</td>
<td>0</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1) Cloud reading @ 660mp</td>
<td>48</td>
<td>48</td>
<td>63</td>
<td>63</td>
</tr>
<tr>
<td>(2) Hesperidin content of methanol extract</td>
<td>161 mg%</td>
<td>161 mg%</td>
<td>162 mg%</td>
<td>164 mg%</td>
</tr>
<tr>
<td>(3) Hesperidin content of aqueous extract</td>
<td>156 mg%</td>
<td>161 mg%</td>
<td>133 mg%</td>
<td>132 mg%</td>
</tr>
<tr>
<td>(4) Crystalline hesperidin content (2-3)</td>
<td>5 mg%</td>
<td>-0</td>
<td>29 mg%</td>
<td>32 mg%</td>
</tr>
<tr>
<td>(5) Crystal count</td>
<td>2</td>
<td>0</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>
The results were substantially similar to those obtained using "unpasteurised" juice with regard to an increase in crystalline hesperidin and the elevation of cloud level. However the major difference between the "pasteurised" and unheated juice was that no crystals were detected in the freshly "pasteurised" juice, whereas some were evident in the freshly expressed juice which had not been heated. The cloud of the "pasteurised" juice was also stable well beyond 72 hrs storage.

(b) Extraction of the juice by reaming or with a hypodermic needle

Juice was extracted from an orange either by inserting a fine hypodermic needle into individual juice sacs or by conventional reaming. The hesperidin content of equivalent volumes of this juice was assayed as described previously, and the number of crystals present in the juice was determined by negative staining. The results obtained from 3 oranges are listed in Table 13. With the syringed samples there was insufficient liquor to obtain a cloud reading.

| TABLE 13 Hesperidin content of juices extracted by reaming or hypodermic syringe |
|-----------------|-----------------|-----------------|-----------------|
| Sample          | Hesperidin in aqueous extract mg% | Hesperidin in methanol extract mg% | Crystal count |
| A               |                              |                              |                |
| Reamed          | 120                          | 123                          | 1              |
| Syringed        | 60                           | 60                           | 0              |
| B               |                              |                              |                |
| Reamed          | 100                          | 105                          | 2              |
| Syringed        | 60                           | 60                           | 0              |
| C               |                              |                              |                |
| Reamed          | 105                          | 110                          | 2              |
| Syringed        | 80                           | 81                           | 0              |
It can be seen that in each sample examined the juice extracted with the syringe always contained substantially less total hesperidin than the equivalent reamed juice. Furthermore, a limited number of hesperidin crystals were always detected in the reamed juice, whereas none were detected in the syringed juice.

(c) Hesperidin content of rag and pulp

Oranges were hand reamed, and then the rag and pulp adhering to the reamer and the extracted half orange "cup" were collected. This material was then extracted with methanol or water for 10 minutes at room temperature either with or without ultrasonic treatment. The hesperidin content of the water and methanol extracts was then determined, and the results obtained are given in Table 14. It can be seen that substantial quantities of hesperidin can be extracted from the rag and pulp. The application of ultrasonic treatment during the extraction greatly enhanced the quantity of hesperidin liberated.

(d) Treatment of fresh juice with hesperidinase

Oranges were hand reamed and the cloud fraction isolated by centrifugation. Aliquots (10 ml) of the juice were then treated with 2 ml of 0.1% hesperidinase (β-glucosidase activity) solution or 2 ml of distilled water and incubated at 30°C for 48 hrs. At the end of this period the cloud level and the crystalline hesperidin content of the samples was determined. The results of a replicate experiment using different batches of oranges are given in Table 15. It can be clearly seen that the treatment with the enzyme has resulted in a less cloudy juice, than the control diluted with an equivalent volume of water. The enhancement of the cloud level of the control over the treated sample was 20% and 11% respectively.
### TABLE 14 Amount of hesperidin extracted from rag and pulp

<table>
<thead>
<tr>
<th>Sample</th>
<th>mg hesperidin/g of rag &amp; pulp</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
</tr>
<tr>
<td>Without ultrasonics</td>
<td>150</td>
</tr>
<tr>
<td>With ultrasonics</td>
<td>250</td>
</tr>
<tr>
<td>B</td>
<td></td>
</tr>
<tr>
<td>Without ultrasonics</td>
<td>250</td>
</tr>
<tr>
<td>With ultrasonics</td>
<td>312</td>
</tr>
</tbody>
</table>

### TABLE 15 Cloud level and hesperidin content of juices treated with hesperidinase

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cloud reading @ 660 mμ</th>
<th>Hesperidin content mg%</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enzyme treated</td>
<td>70</td>
<td>5</td>
</tr>
<tr>
<td>Control</td>
<td>84</td>
<td>15</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enzyme treated</td>
<td>79</td>
<td>2.5</td>
</tr>
<tr>
<td>Control</td>
<td>88</td>
<td>7</td>
</tr>
</tbody>
</table>
However, in both cases a limited amount of crystalline hesperidin was present in the enzyme treated juice and this would indicate that the enzyme had not been totally effective in preventing hesperidin crystallisation.

9. Hesperidin content of Lemon juices

The cloud level and hesperidin content of an aqueous and methanol extract of the cloud fraction of the lemon juices was determined, and the results are given in Table 16.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cloud Level @ 660nm</th>
<th>Hesperidin Content of methanol extract</th>
<th>Hesperidin Content of aqueous extract</th>
<th>Crystalline Hesperidin content methanol minus water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sicilian 400 H.C.</td>
<td>54</td>
<td>280 mg%</td>
<td>140 mg%</td>
<td>140 mg%</td>
</tr>
<tr>
<td>(1973)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sicilian 400</td>
<td>27</td>
<td>50 mg%</td>
<td>50 mg%</td>
<td>0 mg%</td>
</tr>
<tr>
<td>New Sicilian 400 H.C.</td>
<td>95</td>
<td>130 mg%</td>
<td>20 mg%</td>
<td>-110 mg%</td>
</tr>
<tr>
<td>Sicilian 400 H.C. 1974</td>
<td>60</td>
<td>250 mg%</td>
<td>100 mg%</td>
<td>150 mg%</td>
</tr>
</tbody>
</table>

It can be seen that no general correlation between hesperidin crystals and cloud level exists for these four samples. With two of the samples relatively high levels of hesperidin are associated with products of high cloud level. The new Sicilian 400 HC which exhibits an outstandingly high cloud level appears to contain no hesperidin. The examination of this product by light microscopy
indicated that it was virtually entirely composed of oil droplets, and as such was atypical of the three other products.
DISCUSSION

The structural studies performed during the course of this work clearly indicate that considerable information can be obtained from the judicious application of electron microscopy techniques to foodstuffs. However, it is clear that no one single preparative technique was capable of providing all the structural information and it was necessary to combine the results of thin sectioning, negative staining, freeze etching and scanning electron microscopy. It was necessary to use several procedures because the rigours of specimen preparation can lead to the loss of certain constituents from the tissue or the formation of artifacts.

The current techniques for electron microscopy have obviously been evolved to minimise the artifact problem. Nevertheless, there are still difficult areas and these have been experienced during this work. One of the major difficulties in the thin sectioning technique is the fixation and embedding of the sample. The tissue must be rendered insoluble and then suitably embedded in a supporting medium, with the minimum of conformational and structural deformation. The use of glutaraldehyde (Sabatini, Bensch and Barnett, 1963) and osmium tetroxide (Palade, 1952) as fixatives works well for most biopolymers such as proteins, carbohydrates and nucleic acids, but the fixation of lipids is notoriously difficult. Buffered osmium tetroxide is the most effective fixative for lipid available at this time. It is considered that the osmium binds to the unsaturated double bonds in fatty acids to form an osmate ester and so render the fat insoluble (Hofmann, 1912). Other mechanisms for the action of osmium have been considered and an alternative view (Sjostrand, 1959, Stoeckenius, 1959) considers that the osmium becomes associated with the polar end of the lipid. Once the tissue has been fixed the sample
must be dehydrated prior to embedding in a plastic resin. Cope and Williams (1969) have shown, using radio chemically labelled model lipid systems, that in some cases the loss of lipid can be as high as 90% in the dehydration and embedding stages, even after fixation with osmium tetroxide.

Another artifact which is related to botanical specimens is the problem of compression of the cell wall during the cutting of the section. Cox (1969) has shown that the compression marks, which tend to traverse the cell wall, are caused during the cutting action, and are similar in appearance to the plasmadesma which are true structural entities that cross the cell wall. It was found during this project that some of the fresh tissues were very difficult to section. The walls of the albedo and segment membrane frequently contained folds and compression artifacts of the type described above.

Another problem which was specific to this research was the observation that crystalline hesperidin was never observed in any of the sections. This may probably be explained by the fact that hesperidin is readily soluble in alcohol, and thus is probably lost during the dehydration stage of specimen preparation.

With the introduction of the freeze etching technique (Steere, 1957) it was anticipated that artifacts from fixation dehydration and embedding could be avoided. However, as indicated in the introduction to this thesis, although some of the problems associated with thin sectioning can be avoided a whole range of artifacts specific to freeze etching have been recognised. Of these, the two most important problems are probably the formation of ice crystals during the freezing stage, and deposition of contaminants from the vacuum system onto the specimen prior to formation of the replica. If relatively large ice crystals are formed during freezing these may distort or obliterate the fine structure of the sample. This
problem may be minimized by the use of a suitable cryoprotectant, and during the course of this work it was found that a high level of soluble solids (i.e., greater than 44° Brix) functioned as an efficient cryoprotectant. If the surface of the sample becomes covered with contaminants from the vacuum system these may well be interpreted as true structures rather than adhering contaminants. This problem may be minimized by ensuring that the vacuum system is clean and well trapped with liquid nitrogen to preferentially condense the vacuum oil contaminants.

Despite the problems listed above, the freeze etching technique has become a most useful complement to thin sectioning. The results obtained during this investigation have shown that freeze etching is the ideal method for observing the oil droplets and hesperidin crystals found in orange juice, since it is probable that both these constituents will be solubilized during conventional thin sectioning.

The major artifact associated with negative staining is the possible collapse and distortion of the sample during the drying down stage. Another particular problem encountered during this work was the formation of spurious images in the negative stain preparations, due to the volatility of certain constituents deposited onto the specimen grid. However, the negative staining procedure was used in this investigation primarily for the quantitative assessment of the hesperidin content of the samples, and should have proved adequate for such purposes.

The foregoing comments indicate that caution must be exercised when interpreting the results from electron microscopy, and due consideration given to potential artifacts.

The fine structural changes associated with the processing of food derived from plant materials has been studied by relatively few workers. As was mentioned in the introduction, the main systems
studied have been freezing (Bassi and Crivelli 1968; 1969), pickling (Saxton and Jewell, 1969; Jewell 1972b) and more recently blanching (Mohr, 1974). All of these workers have observed the destruction and degradation of cellular organelles, the breakdown of the tonoplast, the rupture of the plasmalemma, and in some instances loss of cell wall constituents. In more conventional botanical materials the loss of fine structure has been observed in studies on senescence, ripening and parasitic infections. The ultrastructural changes associated with senescence of cotyledons and leaves has been documented by amongst others Shaw and Manocha (1965) and Butler (1967). General aspects of senescence in plants have been reviewed by Butler and Simon (1971) and they have concluded that the degeneration and death of the cell follows a characteristic and repeatable pattern. The first detectable changes are usually a decrease in the number of ribosomes and the start of chloroplast breakdown. The free ribosomes are the first to disappear followed by those attached to the endoplasmic reticulum. The chloroplast stoma disappears, the thylakoids swell and disintegrate, and there is a marked increase in the number and size of osmiophilic globules. In some instances the mitochondria show evidence of swelling and a reduction in the number of cristae. The endoplasmic reticulum swells, vesiculates and then disappears, as do the Golgi apparatus. The tonoplast membrane breaks down and eventually the plasmalemma ruptures as well. The nucleus remains relatively stable, but at a late stage the nuclear membrane, vesiculates and breaks down and the chromatin shrinks and disappears.

Similar changes in fine structure were noted by Bain and Mercer (1964) during the ripening of pears. They noted in particular the occurrence of osmiophilic droplets associated with the degradation of chloroplasts. Wrischer (1965) also noted that pressure on plant tissue resulted in an increase in the number of osmiophilic droplets.
in chloroplasts and chromoplasts.

From the discussion above it is obvious that with an intact tissue, the process of the death of the cell may be studied, and a number of well characterised changes observed. However, when the death of the cells occurs by a drastic and relatively rapid procedure such as extracting juice from an orange by reaming, no such gradual changes can be observed. Instead, one must rely on a knowledge of the fresh tissue organisation in an attempt to recognise the origin of the cellular debris in an extracted juice.

The results contained within this thesis indicate that the cells of the flavedo and the outer cells of the juice sac exhibited good cellular preservation. This would indicate that the fixation procedure was adequate. On the basis of the premise of an acceptable fixation regime, the lack of fine structure in the cells of the albedo, segment membrane and innermost cells of the juice sac would tend to suggest that this loss of ultrastructural detail might be related to a general degradation of certain cells during the ripening process.

The observations of particular interest from the thin sections were the structure of the chromoplasts and the nature of the cell walls of some of the cells. The structure of the chromoplast has been described by Fry Wyssling and Muhlethaler (1965) who considered that chromoplasts may be derived from degraded chloroplasts. The characteristic feature of chromoplasts (apart from their colour) when viewed in thin section is the abundance of osmiophilic globules and membrane remnants. This study confirmed that this type of structure was also typical of orange chromoplasts.

The distinctive appearance of the cell walls of the juice sac enabled a precise identification of this material in extracted juice. The outer cell was covered in an osmiophilic cuticle layer and as such could be readily recognised. The fine structure of plant cuticles
has been studied by Chafe and Wardrop (1973), they noted a lamellar appearance of the outer layer of some cuticles and this structure was also observed on certain of the juice sac cuticles. Probably the most striking aspect of the juice sac cell walls were the extremely thin wall structures observed in the inner most cells of the sac. These walls were so thin (10 - 30 nm thick) that initially they were regarded as membranes rather than cell walls. Indeed, the generally accepted dimensions for the unit bimolecular lipid leaflet membrane is 7.5 - 10 nm, with a double membrane being 15 - 20 nm (Robertson, 1959). However, by tracing these walls back to their origin from the somewhat thicker outer cell walls it became obvious that these structures appear to be actual cell wall material. The reason why these walls should be so thin is not at all clear. From their dimensions (i.e.: 10 - 30 nm thick) one would conclude that compared with normal walls (of 0.5 - 2.0 μm thickness) these thin walls would not be capable of great mechanical strength, and as such would provide little structural support for the tissue. Therefore, it seems probable that the main function of these walls is to compartmentalise the juice sac, and as such no great mechanical rigidity would be required.

In contrast to the very thin walls of the juice sac, the walls of the albedo were extremely thick. This somewhat characteristic appearance was again of considerable use in identifying the origin of the various tissues in the comminuted bases. Seen from a different standpoint, if a juice was found to contain a large number of cell walls typical of the albedo, it would indicate that the juice was not strictly speaking genuine since some peel tissue had been added. Thus, the ability to characterise the various tissue types present in juices and bases may be able to make a contribution to the problem of verification of juice authenticity.

The unusual structures observed in the neck rings on the products
were interesting from two standpoints. First, the observation that bases prepared using different processes gave neck rings with different structures. Secondly, the origin of the droplets found in the neck rings. An understanding of the origin of the droplets, might well explain why different processes give different types of droplets. In general terms from their appearance and staining characteristics with osmium tetroxide one would conclude that the droplets are composed of lipid. The type of droplets typified in Plate 49 show a close morphological similarity to the chromoplasts as illustrated on Plate 30. This would tend to indicate that these droplets might be derived from chromoplasts. This postulate would seem reasonable since by their lipid content the density of these droplets would be considerably less than the density of the aqueous phase of the product. And thus on storage they would tend to separate to the top of the container. Furthermore, the visual appearance of the neck rings is frequently of an intensely orange coloured layer and again this would be compatible with the concept that the ring was rich in chromoplast material. The other type of droplet as illustrated in Plate 52, appears to be bounded by a distinct membrane structure. The limiting membrane was confirmed by freeze etching. Relatively few discrete membrane bound droplets were observed in the fresh tissue, although numerous membrane bound vesicles were noted in the extracted juice. Therefore, the neck ring droplets might have originated from the membrane bound vesicles formed during the disintegration of the tissue. It is a fairly common commercial practice during the formulation of drinks to add extra orange oil in order to enhance the flavour of the product. It is therefore feasible, that some of this added oil may become membrane bound either with natural membranes or added emulsifying agents and so produce some neck ring droplets.
As was mentioned earlier in this thesis the precise details of base manufacture are regarded as confidential information. Thus one can only speculate that the differences within the mode of preparation might well give rise to a base which is relatively rich in liberated chromoplast material or a base relatively deficient in free chromoplast material. Whence, products from the former base would contain predominantly chromoplast material in the neck ring, and products from the latter type of base would contain mainly lipid materials other than chromoplasts.

The results from thin sectioning indicated that the cloud material was predominantly cell wall and cellular debris. The freeze etching technique showed that the cloud also contained numerous clusters of small droplets and crystalline material. The dimensions of these clusters of droplets were from 0.5 - 1.0 μm and as such these aggregates are probably the lipid droplets identified by numerous workers using light microscopy (Davis, 1932; Resch and Schara, 1970). These oil globules have been the corner stone of the theory of the cloudiness of orange juice, since it is generally accepted that the cloud is attributable to the globules of greater than 0.5 μm diameter reflecting light. It would therefore seem significant, that when visualised with the electron microscope very few of these structures are in fact single globules but normally clusters of very much smaller droplets.

Therefore, if these aggregates became disrupted and the droplets dispersed, the mean droplet diameter would be considerably below that required to reflect light and the cloud level of the sample would be markedly reduced.

A further interesting result from the freeze etching was the observation that oil droplets are frequently associated with the hesperidin crystals. This might well lead to an increased buoyancy of the crystals and so aid their retention in the cloud fraction of the sample.
An observation from the freeze etching which was difficult to explain was the phenomenon of the cell walls only being apparent in specimens which exhibited ice crystal formation. A possible explanation is that with the formation of the ice crystals the aqueous matrix tended to move away from the wall structures, and since in an icy specimen one can achieve relatively deep etching (as the water vapour pressure is high) the cell walls became visible. In the case of the cryoprotected samples, the ice crystal formation was minimal and as the vapour pressure of the water is lowered by the cryoprotectant the depth of etching is drastically reduced. In this situation one must presume that the profiles of the wall were not discernible.

The major gain obtained from the negative staining was the realisation that the crystal content of the various samples was markedly different. Indeed, from all the microscopical results the only major difference between the samples which might be related to cloud level was the number of hesperidin crystals.

When the crystal content of the samples were plotted against cloud level a linear relationship was obtained. These plots for the juices, bases and products did not pass through the origin on the cloud axis, clearly showing that the crystal content is not the sole factor involved in determining cloud level.

The combination of melting point and ultraviolet spectroscopy techniques on the isolated crystals indicated that the material was hesperidin. The initial attempts to quantify hesperidin were based on the Davis method (Davis, 1947) which is given in Appendix V. The reason this method was adopted was that it had been used by several workers in relatively recent studies (Koch and Hess, 1971; Baker and Bruenmer, 1972). However, the Davis method was originally evolved for the estimation of naringin, the principle flavanone of grapefruit, and is not strictly applicable for the estimation of
hesperidin. This point has been clearly made by Horowitz and Gentili (1959) and again by Hendrickson et al. (1959), and both these groups of workers found that the Davis method gave high estimates for hesperidin.

It was in fact a modification of the method of hesperidin analysis proposed by Hendrickson et al. (1959) that was adopted for use in this work. The modification of the method stemmed from the realisation that it was an estimate of crystalline hesperidin that would be useful for correlation with cloud level. One of the notable features of hesperidin is its marked insolubility in water, and as such it is somewhat atypical of flavanone glycosides which are generally considered to be water soluble. In cold water it is soluble to the extent of 1g in 50 litres, in hot water this increases to 1g in 5 litres (Merck Index, 1968). The most effective solvents for hesperidin are ethanol, methanol, pyridine and sodium hydroxide (Merck Index, 1968). Thus, any method of estimation which is going to utilise these solvents will give a measure of total hesperidin, i.e.: material already in solution and crystalline hesperidin which will have been dissolved. For this reason the method of Hendrickson et al. (1959) was modified so that the stage requiring the addition of sodium hydroxide was omitted. Thus the methanol extract gave an indication of total hesperidin, whilst the aqueous extract gave only hesperidin in solution. The difference of methanol minus water was taken to give crystalline hesperidin. With most samples it was found that the crystalline hesperidin accounted for less than half of the total hesperidin figure. This might be taken to indicate that there was a substantial quantity of hesperidin in solution which had not crystallised. It would seem unlikely that this would be the case, since, if for arguments sake, the readings indicated that 120 mg% were remaining in solution (and recourse to Table 10 will indicate
that this is a conservative estimate) this is well above the solu-

bility limit for both cold and hot water of 2 mg% and 20 mg% res-
pectively. It is of course possible that the solubility of hesperidin
in orange juice might be significantly different from that in water.
The results obtained from the synthetic orange juice indicated that
the solubility was substantially the same as that reported for water
either hot or cold. Whence, if it is unlikely that the substance in
solution is hesperidin we are faced with at least two further
possibilities:

(a) The material is a modified hesperidin which has a
greater solubility and so does not crystallise.

(b) The material is not hesperidin, but a compound which
absorbs at the 285 m\text{\mu} wavelength in the ultraviolet
spectrum.

It has been suggested by Wawra and Webb (1942) that in orange
juice the hesperidin in the flavanone form may be in equilibrium with
the chalcone form. The chalcone form was considered by those authors
to be more soluble and thus might support concept (a) above. How-
ever, whilst it is true that chalcone forms can be produced from
flavanones the equilibrium in an acid environment is in favour of the
flavanone form. Thus, in the acid environment of the juice (pH 3.5)
it would seem likely that the flavanone form would predominate, and
as such contention (a) would seem unfounded.

The findings of Coffin (1971) indicated that orange juice con-
tained three flavanone glycosides, of these one was only present in
trace amounts, whilst the remaining two were present in approximately
equal levels. These two were identified as hesperidin (hesperetin 7
rutinoside) and naringenin 7 rutinoside. Although ultraviolet
spectra for the naringenin 7 rutinoside were not determined during
the course of the project, it would seem highly probable that the
spectra of hesperidin and this compound would be very similar, since
they are both 7 rutinosides (Fig. 14) and the spectra for the aglycones hesperetin and naringenin are virtually identical (Sinclair, 1961). Thus, it would appear that the methanol extract is not giving an estimation of hesperidin alone as suggested by Hendrickson et al. (1959), but more probably hesperidin and naringenin 7 rutinoside. This would tend to support proposal (b). Therefore, the best estimate of hesperidin is obtained by using the difference between the methanol and water extracts, and total hesperidin is only likely to be a few more mg% greater than the figure obtained for the crystalline form.

The observation that the turbidity of freshly expressed juice increases upon ageing was first reported by Loeffler (1941), but Mizrahi and Berk (1970) were the first to suspect that this might be related to hesperidin crystallisation, and that the flavanones might play an important part in juice cloudiness. Baker and Bruemmer (1972) dismissed this concept as peculiar to the Shamouti variety of orange, whereas the results contained in this thesis would indicate that flavanone crystallisation is a significant factor in cloud level of juices derived from several varieties of oranges.

The reason why so many workers have paid little attention to the role of hesperidin is probably explained by the fact that the majority of the crystals cannot be observed in the light microscope, since their dimensions in two planes are normally less than 0.25μm which is the resolution limit of light microscopy. The exception to this statement will be when the crystals form characteristic spherulites whose dimensions are adequate to be seen by light microscopy. The work on important cloud particles by Scott et al. (1965) and Baker and Bruemmer (1969) fractionated the cloud using various solvents and the fraction which was removed with alcohol was discarded. It is highly probable that the bulk of the hesperidin would have been
Fig. 14. Structure of (a) hesperidin (hesperetin 7-rutinoside) and (b) naringenin 7-rutinoside.
present in this fraction, and so neglected. Coupled with the lack of interest in hesperidin it is interesting to note that a typical hesperidin content of fresh juice would be of the order of 50 - 100 mg% (See Table 10) whereas the oil content of juice is only 6 - 10 mg% (Kirchner & Miller, 1957).

In a recent study on cloud stability using the concepts of colloid theory, Lankveld (1973), showed that flocculation of the cloud particles was the main factor causing separation and clarification. He considered that during the early stages of flocculation a nett increase in turbidity might result from the increased particle size. In contrast, Baker and Bruemmer (1972) indicated that as a floc forms in juice so the turbidity decreases. These workers analysed the floc and found it to be composed of pectin and hesperidin. At first sight this might indicate that hesperidin has a clarifying role rather than a clouding function. However, these workers used "unpasteurised" juice so that the mechanism of flocculation probably involved formation of insoluble pectates which occluded the crystalline hesperidin. Lankveld (1973) also concluded that the refractive index of the dispersed phase was a significant factor and calculated that in the case of lemon juice the most important particles would have a refractive index of at least 1.48. He surmised that the most optically dense component in the system would be the citrus oil. The results contained within this thesis have shown that the refractive index for hesperidin is 1.59 and so on the basis of this, hesperidin should play a significant role in juice cloudiness.

A simple way to have confirmed the role of hesperidin in cloud level would have been to have taken a juice of given cloud level, dissolve the hesperidin and remeasure the cloud level. Unfortunately, this direct approach was not possible. As was mentioned earlier, the
The most effective solvents for hesperidin are alcohols, pyridine and sodium hydroxide. The addition of any of these solvents to a juice caused a rapid flocculation to occur, presumably due to denaturation of the pectin. Thus, a less direct route was evolved whereby the action of hesperidinase (β-glucosidase activity) on freshly extracted juice reduced the amount of crystalline hesperidin formed and so produced a juice of markedly lower cloud level than the control sample.

Although the bulk of the work contained within this thesis has been aimed at orange juice, a limited amount of work was undertaken on lemon juice. According to Coffin (1971) there are 3 major flavanone glycosides in lemon juice, namely, hesperidin, naringenin 7-rutinoside and eriodictyol 7-rutinoside. Of these three it is again the hesperidin which has the markedly low solubility and is therefore the most likely material to be present in a crystalline form. Therefore, it was interesting to investigate whether hesperidin played such an important role in lemon juice cloud. The results from the examination of 4 samples were not conclusive. The very cloudy lemon juice appeared to contain no hesperidin and was microscopically atypical. Of the remaining 3 samples there appeared to be a relationship between hesperidin content and cloud level. Clearly, further work would be necessary to establish the significance of hesperidin levels in lemon products.

The most recent studies in citrus juice stability have again been aimed at the role of pectin in cloud stabilisation. Thus, Krop and Pilnik (1974a, b) have been studying the effect of enzymes such as polygalacturonase and protease on cloud stability. Whilst not wishing to detract from the importance of pectin in citrus juices, the results contained within this thesis would seem to indicate that in the case of orange juice, considerable commercial advantage could
be obtained from studying methods of maximising the occurrence of crystalline hesperidin in the juice.
REFERENCES


BERGER, K.G., BULLIMORE, B.K., WHITE, G.W. and WRIGHT, W.B. (1972). Dairy Industries 37 (8) 419 and 37 (9) 493.


AN ELECTRON MICROSCOPY STUDY
OF THE PARTICULATE MATERIAL IN
CITRUS FRUITS AND BEVERAGES.

by

GERRARD GRANVILLE JEWELL M.I.Biol.

Thesis submitted in partial fulfilment
of the requirements for the degree of
Ph.D. of the University of Surrey.

Research conducted at
The Food Research Association,
Leatherhead, Surrey.

Submitted: June 1975.
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ABBREVIATIONS USED ON THE PLATES

A  -  cytoplasm
B  -  cuticle
C  -  cell wall
D  -  debris
E  -  electron opaque precipitate
F  -  pit-field
G  -  nuclear membrane
H  -  membrane bound vesicle
I  -  intercellular space
J  -  junction point
K  -  granular body
L  -  lipid droplet
M  -  nucleolus
N  -  nucleus
O  -  chromoplast
P  -  plasmadesmata
R  -  oil gland
S  -  densely staining particle
V  -  vacuole
X  -  crystal
Z  -  smooth zone
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<td>TREATMENT</td>
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<td>Shape and comparative size of the cells.</td>
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<th>PLATE 2</th>
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<td>SAMPLE</td>
<td>Transverse section of flavedo.</td>
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<td>TREATMENT</td>
<td>Cryostat section. Polarised light illumination.</td>
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<td>NOTE</td>
<td>Birefringent nature of cell walls and crystals (X) in some cells.</td>
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PLATE 3
SAMPLE  Transverse section of flavedo.
TREATMENT  Cryostat section.  Phase contrast illumination.
NOTE  Oil gland (R).

PLATE 4
SAMPLE  Transverse section of flavedo.
TREATMENT  Cryostat section.  Polarised light illumination.
NOTE  Birefringent nature of cell walls and oil gland (R).
PLATE 5

SAMPLE  Transverse section of albedo.
TREATMENT  Cryostat section. Stain - safranin/fast green.
NOTE  Thick cell walls.

PLATE 6

SAMPLE  Section of albedo.
TREATMENT  Cryostat section. Safranin/fast green.
NOTE  Thickened cell walls surrounding vascular cells (arrow).
PLATE 7

SAMPLE   Section of albedo.

TREATMENT Cryostat section. Polarised light illumination.

MAGNIFICATION x 450

PLATE 8

SAMPLE   Section of juice sac.

TREATMENT Cryostat section. Polarised light illumination.

MAGNIFICATION x 1,800
PLATE 9  MAGNIFICATION x 4,000
SAMPLE  Israeli 66° Brix juice.
TREATMENT Drop dispersed on slide. Phase contrast illumination.
NOTE  Nucleus (N) surrounded by chromoplasts (0).

PLATE 10  MAGNIFICATION x 4,000
SAMPLE  Spanish base.
TREATMENT Drop dispersed on slide. Phase contrast illumination.
NOTE  Spherulite cluster of crystals (X).
PLATE 11

SAMPLE  Israeli 66° Brix juice.
TREATMENT Drop dispersed on slide. Bright field illumination.
NOTE  Chromoplasts (0), crystals (X) and cellular debris.

PLATE 12

SAMPLE  Product white cap.
TREATMENT Drop dispersed on slide. Dark field illumination.
NOTE  Mass of crystals.
PLATE 13

SAMPLE  Thin section of flavedo.

TREATMENT  Fixation - Glutaraldehyde/osmium tetroxide.
            Stain - Uranyl acetate/lead citrate.

NOTE  Plasmadesma (P) traverse the cell wall (C).  Nuclei (N)
      are present in the densely staining cytoplasm.
PLATE 14

SAMPLE  Thin section of flavedo.

TREATMENT  Fixation - Glutaraldehyde/osmium tetroxide.
             Stain - uranyl acetate/lead citrate.

NOTE  Nucleus (N) and densely staining lipid droplets (L).

MAGNIFICATION x 16,000
PLATE 15  MAGNIFICATION x 10,000

SAMPLE  Thin section of flavedo.

TREATMENT  Fixation - glutaraldehyde/osmium tetroxide.
            Stain - uranyl acetate/lead citrate.

NOTE  Central vacuole (V), pit-field (F) in cell wall (C),
      intercellular space (I) and lipid droplets (L).
SAMPLE: Thin section of albedo.

TREATMENT: Fixation - glutaraldehyde/osmium tetroxide.
Stain - uranyl acetate/lead citrate.

NOTE: Plasmadesma (P) traverse the cell wall at a pit-field (F).
SAMPLE
Thin section of albedo.

TREATMENT
Fixation - glutaraldehyde/osmium tetroxide.
Stain - uranyl acetate/lead citrate.

NOTE
The cells contain a large vacuole (V) with a thin layer of cytoplasm (A) lying against the cell wall (C).
PLATE 18

SAMPLE Thin section of albedo.

TREATMENT Fixation - glutaraldehyde/osmium tetroxide.
Stain - uranyl acetate/lead citrate.

NOTE Cell walls (C) and intercellular spaces (I).
Sample: Thin section of albedo.

Treatment: Fixation - glutaraldehyde/osmium tetroxide.  
Stain - Uranyl acetate/lead citrate.

Note: Thick cell walls (C) and intercellular spaces (I).
SAMPLE Thin section of segment membrane.

TREATMENT Fixation - glutaraldehyde/osmium tetroxide.
Stain - uranyl acetate/lead citrate.

NOTE Cell walls, cytoplasm (A) and vacuoles (V).
PLATE 21

MAGNIFICATION x 5,000

SAMPLE
Thin section of segment membrane.

TREATMENT
Fixation - glutaraldehyde/osmium tetroxide.
Stain - uranyl acetate/lead citrate.
SAMPLE  Thin section of segment membrane.

TREATMENT  Fixation - glutaraldehyde/osmium tetroxide.  
            Stain - uranyl acetate/lead citrate.

NOTE  Fibrous appearance of the cell walls, and electron opaque precipitate (E) deposited predominantly on the walls.
SAMPLE
Thin section of juice sac.

TREATMENT
Fixation - glutaraldehyde/osmium tetroxide.
Stain - uranyl acetate/lead citrate.

NOTE
Cuticle (B) covers outer layer of columnar cells.
Vacuoles (V) and lipid droplets (L) present within cytoplasm.
PLATE 24

SAMPLE  Thin section of juice sac.

TREATMENT  Fixation - glutaraldehyde/osmium tetroxide.
            Stain - uranyl acetate/lead citrate.

NOTE  Vacuoles (V) and lipid droplets (L).
SAMPLE  Thin section of juice sac.

TREATMENT  Fixation - glutaraldehyde/osmium tetroxide.
Stain - uranyl acetate/lead citrate.

NOTE  Very thin cell walls (C).
<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>Thin section of juice sac.</th>
</tr>
</thead>
</table>
| TREATMENT | Fixation - glutaraldehyde/osmium tetroxide.  
Stain - uranyl acetate/lead citrate. |
| NOTE | The thin cell walls (C) may be traced back to a junction point (J) with thicker cell walls. |
SAMPLE Thin section of juice sac.

TREATMENT Fixation - glutaraldehyde/osmium tetroxide.  
Stain - uranyl acetate/lead citrate.

NOTE The cuticle (B) on the outer cell of the juice sac.
SAMPLE  Thin section of juice sac.

TREATMENT  Fixation - glutaraldehyde/osmium tetroxide.
            Stain - uranyl acetate/lead citrate.

NOTE  The debris (D) adhering to the cuticle (B).
SAMPLE  Thin section of juice sac.

TREATMENT  Fixation - glutaraldehyde/oxmium tetroxide.  
           Stain - uranyl acetate/lead citrate.

NOTE  Pit-field (F) with plasmadesmata (P).  Nucleus (N) 
       with nucleolus (M) and nuclear membrane (G).  
       Vacuole (V), lipid droplet (L) and chromoplast (O).
SAMPLE
Thin section of juice sac.

TREATMENT
Fixation – glutaraldehyde/osmium tetroxide.
Stain – uranyl acetate/lead citrate.

NOTE
The chromoplasts (0) contain circular arrays of lipid droplets (L).
**SAMPLE**  Thin section of juice sac.

**TREATMENT**  Fixation – glutaraldehyde/osmium tetroxide.  
Stain – uranyl acetate/lead citrate.

**NOTE**  Thin cell walls with little evidence for pit-fields or plasmadesma.
SAMPLE Thin section of juice sac.

TREATMENT Fixation - glutaraldehyde/osmium tetroxide.
Stain - uranyl acetate/lead citrate.

NOTE Membrane bound vesicles (H) and lipid droplet (L).
SAMPLE Thin section of juice sac.

TREATMENT Fixation - glutaraldehyde/osmium tetroxide.
                         Stain - uranyl acetate/lead citrate.

NOTE Lipid droplets (L) and aggregates of vesicles (H).
SAMPLE  Thin section of single strength Israeli juice.
TREATMENT  Microencapsulated. Fixation - glutaraldehyde/osmium tetroxide. Stain - uranyl acetate/lead citrate.
NOTE  Cell wall (C) with cuticle (B) and cellular debris (D).
SAMPLE  Thin section of single strength Israeli juice.


NOTE  Aggregates of vesicles (H) and lipid droplets (L).
SAMPLE
Thin section of single strength Israeli juice.

TREATMENT

NOTE
Membranes, vesicles (H) and granular bodies (K).
SAMPLE  Thin section of a sixfold Israeli juice.

TREATMENT  Microencapsulated.  Fixation - glutaraldehyde/osmium tetroxide.  Stain - uranyl acetate/lead citrate.

NOTE  Cell walls (C), lipid droplets (L) and chromoplast (O).
<table>
<thead>
<tr>
<th>PLATE 38</th>
<th>MAGNIFICATION x 15,000</th>
</tr>
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<td><strong>SAMPLE</strong></td>
<td>Thin section of a six fold South African juice.</td>
</tr>
<tr>
<td><strong>TREATMENT</strong></td>
<td>Microencapsulated. Fixation - glutaraldehyde/osmium tetroxide. Stain - uranyl acetate/lead citrate.</td>
</tr>
<tr>
<td><strong>NOTE</strong></td>
<td>Cell walls (C) and cellular debris (D).</td>
</tr>
</tbody>
</table>
SAMPLE  Thin section of a six fold Israeli juice.
TREATMENT  Microencapsulated. Fixation - glutaraldehyde/osmium tetroxide. Stain - uranyl acetate/lead citrate.
NOTE  Cell walls and densely staining particles (S).
SAMPLE  Thin section of a sixfold South African juice.

TREATMENT  Microencapsulated.  Fixation - glutaraldehyde/osmium tetroxide.  Stain - uranyl acetate/lead citrate.

NOTE  Densely staining particles (S) associated with vesicles (H).
SAMPLE  Thin section of a sixfold South African juice.

TREATMENT  Microencapsulated.  Fixation - glutaraldehyde/osmium tetroxide.  Stain - uranyl acetate/lead citrate.

NOTE  Cell wall with cuticle (B) and cellular debris (D).
SAMPLE  Thin section of an Israeli base.

TREATMENT  Microencapsulated.  Fixation - glutaraldehyde/osmium tetroxide.  Stain - uranyl acetate/lead citrate.

NOTE  Thick and thin cell walls.
<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>Thin section of a Spanish base.</th>
</tr>
</thead>
<tbody>
<tr>
<td>TREATMENT</td>
<td>Microencapsulated. Fixation - glutaraldehyde/osmium tetroxide. Stain - uranyl acetate/lead citrate.</td>
</tr>
<tr>
<td>NOTE</td>
<td>Thick and thin cell walls.</td>
</tr>
</tbody>
</table>
SAMPLE  Thin section of a Spanish base.

TREATMENT  Microencapsulated. Fixation - glutaraldehyde/osmium tetroxide. Stain - uranyl acetate/lead citrate.

NOTE  Thin cell walls (C), lipid droplets (L) and densely staining particles (S).
SAMPLE  Thin section of the sediment from a product.


NOTE  Thick cell walls (C) and cellular debris (D).
<table>
<thead>
<tr>
<th><strong>PLATE 46</strong></th>
<th>MAGNIFICATION x 17,500</th>
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<tr>
<td><strong>SAMPLE</strong></td>
<td>Thin section of the sediment from a product.</td>
</tr>
<tr>
<td><strong>TREATMENT</strong></td>
<td>Microencapsulated. Fixation - glutaraldehyde/osmium tetroxide. Stain - uranyl acetate/lead citrate.</td>
</tr>
<tr>
<td><strong>NOTE</strong></td>
<td>Cell walls and cellular debris.</td>
</tr>
</tbody>
</table>
SAMPLE  Thin section of the cloud from a product.

TREATMENT  Microencapsulated. Fixation - glutaraldehyde/osmium tetroxide. Stain - uranyl acetate/lead citrate.

NOTE  Lipid droplets (L) and granular material.
SAMPLE  Thin section of the cloud from a product.


NOTE  Cell wall (C) and cellular debris (D).
<table>
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<th>PLATE 49</th>
<th>MAGNIFICATION x 20,000</th>
</tr>
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<td>SAMPLE</td>
<td>Thin section of the neck ring from a product.</td>
</tr>
<tr>
<td>NOTE</td>
<td>Overall appearance and internal structure of particles.</td>
</tr>
</tbody>
</table>
SAMPLE     Thin section of the neck ring from a product.

TREATMENT  Microencapsulated. Fixation - glutaraldehyde/osmium tetroxide. Stain - uranyl acetate/lead citrate.

NOTE       Internal organisation of the particles.
PLATE 51  MAGNIFICATION x 32,000

SAMPLE  Thin section of the neck ring from a product.

TREATMENT  Microencapsulated. Fixation - glutaraldehyde/osmium tetroxide. Stain - uranyl acetate/lead citrate.

NOTE  Granular bodies (K) and other materials within the particle.
SAMPLE  Thin section of the neck ring from a product.

TREATMENT  Microencapsulated. Fixation - glutaraldehyde/osmium tetroxide. Stain - uranyl acetate/lead citrate.

NOTE  Membrane bound particles.
SAMPLE  Thin section of the neck ring from a product.

TREATMENT  Microencapsulated.  Fixation - glutaraldehyde/osmium tetroxide.  Stain - uranyl acetate/lead citrate.

NOTE  Membrane bound particles.
SAMPLE  Freeze etch replica of an Israeli single strength juice.

TREATMENT  No cryoprotectant.  Etched at -100°C for 2 minutes.

NOTE  Icey matrix, cell walls (C) and droplets.
PLATE 55  MAGNIFICATION x 35,000

SAMPLE  Freeze etch replica of an Israeli single strength juice.
TREATMENT  No cryoprotectant. Etched at -100°C for 2 minutes.
NOTE  Cell walls (C) and crystals (X).
PLATE 56

SAMPLE Freeze etch replica of an Israeli single strength juice.

TREATMENT Juice contains 30% glycerol etched at -100°C for 2 minutes.

NOTE Aggregates of droplets and some crystals (X).
SAMPLE  Freeze etch replica of an Israeli base.

TREATMENT  No cryoprotectant. Etched at -100°C for 2 minutes.

NOTE  Aggregates of droplets and some crystals (X).
SAMPLE: Freeze etch replica of a Spanish base.

TREATMENT: No cryoprotectant. Etched at -100°C for 2 minutes.

NOTE: Aggregate of droplets.
<table>
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<th>SAMPLE</th>
<th>Freeze etch replica of an Israeli base.</th>
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<tbody>
<tr>
<td>TREATMENT</td>
<td>No cryoprotectant. Etched at -100°C for 2 minutes.</td>
</tr>
<tr>
<td>NOTE</td>
<td>Droplets and crystalline material (X).</td>
</tr>
</tbody>
</table>
SAMPLE  Freeze etch replica of an Israeli base.

TREATMENT  No cryoprotectant. Etched at -100°C for 2 minutes.

NOTE  Crystals and layer growth steps (arrow).
SAMPLE  Freeze etch replica of an Israeli 44° Brix juice.

TREATMENT  No cryoprotectant. Etched at -100°C for 2 minutes.

NOTE  Aggregate contains droplets, crystals and granular bodies (K).
SAMPLE  Freeze etch replica of an Israeli base.
TREATMENT  No cryoprotectant. Etched at -100°C for 2 minutes.
NOTE  Aggregate contains droplets, crystals, granular bodies and smooth zones (Z).
SAMPLE: Freeze etch replica of a neck ring from a product.

TREATMENT: No cryoprotectant. Etched at -100°C for 2 minutes.

NOTE: Reticular surface of the droplets.
SAMPLE  Freeze etch replica of a neck ring from a product.

TREATMENT  No cryoprotectant. Etched at $-100^\circ C$ for 2 minutes.

NOTE  Interior of particle appears to contain droplets.
SAMPLE  Freeze etch replica of a neck ring from a product.
TREATMENT  No cryoprotectant. Etched at -100°C for 2 minutes.
NOTE  Droplet appears to be bounded by a membrane (arrow).
PLATE 66

SAMPLE Negative stain preparation of an Israeli single strength juice.

TREATMENT Cloud fraction mixed with 2% phosphotungstic acid pH 5.0.
SAMPLE  Negative stain preparation of an Israeli single strength juice.

TREATMENT  Cloud fraction mixed with 2% phosphotungastic acid pH 5.0.

NOTE  Crystals between circular structures.
SAMPLE  Negative stain preparation of an Israeli single strength juice.

TREATMENT  Sediment fraction mixed with 2% phosphotungstic acid pH 5.0.

NOTE  Crystals are lying upon densely stained material.
SAMPLE  Negative stain preparation of an Israeli single strength juice.

TREATMENT  Sediment fraction mixed with 2% phosphotungstic acid pH 5.0.

NOTE  Fibrillar material (arrow) lying between the crystals.
PLATE 70

SAMPLE  Negative stain preparation of an Israeli 44° Brix juice.

TREATMENT  Cloud washed twice, then mixed with 2% phosphotungstic acid pH 5.0.
SAMPLE

Negative stain preparation of an Israeli base.

TREATMENT

Cloud washed twice, then mixed with 2% phosphotungstic acid pH 5.0.
<table>
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<th>SAMPLE</th>
<th>Negative stain preparation of a Spanish base.</th>
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</thead>
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<tr>
<td>TREATMENT</td>
<td>Cloud washed twice, then mixed with 2% phosphotungstic acid pH 5.0.</td>
</tr>
<tr>
<td>NOTE</td>
<td>Crystals (X) and membrane bound vesicles (H).</td>
</tr>
</tbody>
</table>
Negative stain preparation of a South African juice.

Cloud washed twice, then mixed with 2% phosphotungstic acid pH 5.0.
SAMPLE  Negative stain preparation of a Sicilian lemon juice.

TREATMENT  Cloud washed twice, then mixed with 2% phosphotungstic acid pH 5.0.

NOTE  Large and small crystals.
PLATE 75

SAMPLE  Scanning electron micrograph of cloud particles.

TREATMENT  Cloud dried down onto stub and rotary coated with carbon and palladium.

NOTE  Mass of crystals.

PLATE 76

SAMPLE  Scanning electron micrograph of inner surface of juice sac.

TREATMENT  Juice sac freeze dried onto stub and rotary coated with palladium.

NOTE  Wrinkled appearance of surface.