

Citrus Nutrition and Quality

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FOREWORD

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PREFACE

There is a long romantic history of man's fascination, esteem, and food recognition of citrus fruits. According to one legend, it was not with an apple that Eve tempted Adam but with a citrus fruit (a primitive citron called the etrog or Adam's apple). Oranges were also once considered the fruit of the gods. The ancient dynasties of China regarded citrus fruits as prized tributes. During the reign of Ta Yu (about 2205–2197 B.C.), tributes of mandarins and pummelos, wrapped in ornamental silks, were sent to the imperial court of Ta Yu when specifically ordered.

The importance of citrus fruits and their products as human food is underscored by the fact that more citrus is consumed than any other kind of fruit. To illustrate this point, a recent survey conducted by the Market Research Corporation of America showed that about 68% of all juices (combined total of fruit and vegetable juices) consumed in the United States were citrus juices. The world's citrus crop for the 1979–80 season was estimated at 39.5 million metric tons (MMT), of which the United States contributed a total of 14.4 MMT (oranges 10.4 MMT, grapefruit 2.5 MMT, lemons 0.7 MMT, tangerines 0.5 MMT, other citrus cultivars 0.3 MMT). The citrus industry in Florida is the largest and encompasses 336,384 hectares (about 2/3 of the total U. S. hectareage). During the 1978–79 season, Florida produced 10.1 MMT of citrus fruit with a fresh and processed sales value of \$1.99 billion. World production figures show that the citrus crop is second only to the grape crop. However, most grapes are utilized primarily in fermented liquors rather than consumed as fresh fruit or juice products.

As citrus fruits and their products contribute substantially to the American diet and are consumed in great abundance, we believe the time is appropriate to review in detail some important nutritional and quality properties of this important fruit. Twenty-eight scientists joined with us to cover extensively subjects in the following areas: nutrition and health; quality as related to specific biochemical components; effects of handling and processing; quality control and evaluation; regulatory implication; and adulteration.

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July 14, 1980

Nutrients and Nutrition of Citrus Fruits

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All foods for humans must provide some life-sustaining elements. These elements are the nutrients that supply energy after being metabolized in the body and those that are essential for the body to carry on this metabolism. Other qualifications for a food are its psychological and social effects. Many foods are eaten as a habit, custom, or tradition; but all foods must possess acceptable physical attributes, i.e., color and texture, and desirable taste and palatability.

The acceptance of fruits as a staple in human diet has only been practiced since the past century because of their perishability as fresh produce. In most instances, they are used as desserts because most of them are sweet in taste and because of their high economic values. With the advent of canning and other preservation industries and with the better knowledge of nutrition, the use of fruits as staple foods has become more prevalent, especially in developed countries.

The use of citrus fruit, especially oranges, dramatically increased in the U.S. after World War II (1) because of the introduction of frozen concentrated orange juice (FCOJ) to the market. Citrus fruits, being subtropical products, did not enjoy the popularity of other fruits, e.g. apples, because the locality of production were usually not near the world population centers and because of perishability of citrus fruits during storage. Their susceptibility to physiological disorders and to storage diseases, especially molds and rots, made the cost a deterring factor to consumers. These shortcomings were overcome with the development of FCOJ, which is attractive in color, possesses full fresh orange flavor and greatly reduces the cost of transportation with nearly no storage loss. Research in the area of storage disease control and in transportation of fresh fruit have also led to increased consumption in many developed countries.

Citrus fruits and their products are important sources of vitamin C in the American diet, and are becoming increasingly more important to other developed and developing countries. Consumer awareness of the healthful aspects of citrus, together

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with its appealing color and delightful aroma and taste, makes citrus products the most popular of the processed fruit products. The improved technology of citrus fruit production, processing, storage and transportation has placed the product within economic reach of more people than ever. The cost of a serving (6 fl oz, 177 ml) of orange juice, grapefruit juice, and other juices were 9.7, 9.0 and 10.7 cents, respectively, as reported in September 1979 (2). Table I shows the gallonage and expenditures for citrus fruit juices and fruit-flavored drinks, including orange flavored, in the U.S. during September.

Table I. Consumption of Fruit Juices and Other Fruit Beverages in the United States and Their Total Values-September, 1979

	Millions of gallons	Millions of dollars
Orange juice	47.0	103.7
Grapefruit juice	6.2	12.5
All other fruit juices	26.1	70.0
Orange flavor drinks	11.6	16.2
All other flavor drinks	18.4	26.2

Source: (2)

The Historical Role of Citrus Fruit in the Human Diet

Citrus is generally regarded as one of the most important sources of ascorbic acid. The relation between citrus fruit and antiscorbutic activity actually was first reported by a Hungarian physician, Kramer, in 1732 (3). The dreadful disease of scurvy was found to be completely prevented with the ingestion of green vegetable or pulp of citrus fruit. The use of lime and orange in the diet of seamen in the Royal British Admiralty was the origin of the name "Limey" for British sailors. Harden and Zilva (4) found a concentrated fraction from the lemon fruit that possessed strong reducing properties. Later Szent Gyorgyi (5) isolated the antiscorbutic substance in crystalline form from pepper and citrus fruit.

Many vegetables and fruits, other than citrus, contain ascorbic acid. It was estimated that citrus fruits and tomatoes provided only 18% of the total vitamin C intake in the American diet during the decade of 1910. These two fruits supplied 41% of vitamin C in 1956-58 (6). Today orange juice alone provides nearly 60% of the U.S. Recommended Daily Allowance (U.S. RDA) of vitamin C in the American diet (7).

Citrus fruits and their products are now recognized as an important food in the human diet, not only because of their vitamin C contents, but also because of their other food attributes, such as their pleasant aroma, appealing color, and pleasant taste of appropriate ratios of sweetness and tartness

and because of the awareness by the public of their nutritive values and the other nutrients they contain.

Macronutrients in Citrus

The energy-supplying nutrients are generally carbohydrates, protein and fat. While citrus products provide little protein and fat, their contribution of carbohydrate is an essential part of the nutritive value of citrus. The proximate composition of several kinds of citrus fruits (8) are shown in Table II. Because

Table II. Proximate Composition of Citrus Fruits (g/100 g)

Fruits	Moisture	Protein	Fat	Carbohydrate		Ash
				Sol.	Insol.	
<u>Orange</u>						
Whole fruit	86.4	.7	.2	12.0	.5	.7
Juice	88.3	.6	.2	10.5	.1	.4
<u>Grapefruit</u>						
Whole fruit	88.9	.5	.1	10.1	.2	.4
Juice	90.2	.5	.1	9.0	--	.2
Segment	91.3	.6	.1	7.6	.2	.4
<u>Tangerine</u>						
Whole fruit	87.0	.8	.2	11.6	.5	.4
Juice	88.9	.5	.2	10.1	.1	.3

Source: (8)

the compositions vary greatly due to fruit maturity and variety, these values can fluctuate considerably from actual samples. The main part of the caloric values supplied by citrus is from carbohydrate and most of the protein value is actually from free amino acids.

Carbohydrates

Simple sugars. The main portion of carbohydrates in citrus fruit are the three simple sugars: sucrose, glucose and fructose (9). Together they represent about 80% of the total soluble solids of orange juice (10), and the ratios of sucrose: glucose: fructose are generally about 2:1:1 (11). In over-mature early and mid-season Florida oranges, and in tangerines, the ratios of sucrose to reducing sugars have been found to increase but not in the late season Florida oranges (12). In grapefruit, the sucrose to non-reducing sugar ratios are less than 1. Most of the free sugars in lemon and lime juices are reducing sugars (Table III) and the main soluble solid in these fruit juices is citric acid. In an acidic medium such as citrus juices, sucrose can be easily hydrolyzed; this fact may account for the low sucrose values sometimes found in canned juices subject to long term storage.

Table III. Average Sugar Composition of Citrus Juices (g/100 g)

Fruit	Glucose	Fructose	Total		Total Sugars
			Reducing	Sucrose	
Orange	2.03	2.48	4.51	4.81	9.32
Grapefruit	1.66	1.75	3.41	2.56	5.97
Tangerine	1.13	1.54	2.67	6.53	9.20
Lemon	1.40	1.35	2.75	0.41	3.16
Lime			3.48	0	3.48

Source: (12)

The main sugars in the peel of citrus fruits are also sucrose, glucose and fructose, although a trace of free xylose was reported (13). The relative amounts of these sugars are shown in Table IV. These free sugars are also the major ingredients of cattle feed manufactured from citrus peel and processing residue, especially when citrus molasses is blended into the feed (14).

Table IV. Average Sugar Composition in Citrus Peel

Fruit and variety	Time of harvest	Glucose	Fructose (% dry weight)	Sucrose	Total sugar
Pineapple oranges	January	10.8	21.2	13.9	45.8
Valencia oranges	April	10.9	10.9	16.5	37.4
Marsh seedless grapefruit	December	11.6	12.8	14.3	38.7

Source: (13)

Polysaccharides and Polyuronides. These compounds are found in the alcohol-insoluble fraction and consist of pectic substances, hemicellulose, cellulose and lignin. The recent interest of dietary fiber in human nutrition has placed special emphasis upon these substances in foods. Between 45 and 75 percent of the total solids in citrus peel and membrane is not soluble in alcohol (13), and most of these alcohol insoluble solids consisted of polysaccharides or polyuronides (15). In orange fruit, the peel is not generally eaten except in such speciality products as candied peel or marmalade. The bitterness in the peel and the segment membrane of grapefruit is due to naringin and limonin (12) and makes that portion of the fruit unpalatable. Roe and Bruemmer (16) developed a method to debitter the albedo by vacuum infusion of this tissue with naringinase, thus, rendering the entire fruit, except the flavedo, edible. A distribution of the various component parts of different citrus fruit is shown in Table V (17, 18). Approximately 20 percent of the weight of orange and 30 percent of that of grapefruit is peel (includes both

the albedo and flavedo), and about 10 percent of the weight of the fruit is segment membrane.

Table V. Distribution of the Components of Orange and Grapefruit (% Fresh Weight)

Fruit	Peel	Segment Membrane	Juice vesicles	Seeds
Oranges				
Pineapple	19.9	13.7	62.6	3.8
Valencia	19.2	9.0	71.0	0.8
Grapefruit				
Seedy	27.2	10.0	59.4	3.4

Source: (17, 18)

About 30 percent of the polysaccharides in the peel and pulp may be classified as cellulose (Table VI). No separate cellulose fraction can be distinguished from the juice polysaccharide. Over 50 percent of the total polysaccharide in the peel, 60 percent or more of that of the pulp and over 90 percent of that of the juice are extracted with the pectic substance, the balance being hemicellulose. Separate hydrolysis of these fractions, indicated that some monosaccharides, such as arabinose and galactose, were found in all fractions. Xylose occurred mostly in the hemicellulose fraction whereas galacturonic acid and glucose were the main monosaccharides in the pectic substances and the cellulose fractions, respectively (Table VII).

Dietary Fiber. The definition of dietary fiber is not very specific and it generally includes that group of substances found in the alcohol-insoluble fractions of citrus fruits. The crude fiber value as conventionally reported is only that portion of the dietary fiber consisting of partially purified cellulose and lignin. Although these compounds are not attacked by the human digestive system as they travel through the alimentary tract, they are subject to partial hydrolysis by the microflora in the lower part of the digestive system. The benefit of dietary fiber has been attributed to its ability to decrease the transit time of food through the gastrointestinal tract (19). Some fraction of the dietary fiber such as pectin has been associated with the property of lowering the cholesterol in mammals (20), and the methyl content of the polymer has been reported to be correlated to this capability. Citrus pectin has a methyl content of 7-10 percent as compared to 6-9 percent for apple pectin. Pectin from fleshy fruits such as strawberry has only 0.2 percent (21).

The polysaccharides of the peel and pulp of citrus fruits

Table VI. Percentage Distribution of Various Polysaccharide Fractions in Different Component Parts of Citrus Fruit

Fruit and cultivar	Pectic substance		Hemicellulose		Cellulose		Other					
	Peel	Pulp Juice	Peel	Pulp Juice	Peel	Pulp Juice	Peel	Pulp Juice				
Oranges												
Hamlin	55.4	68.0	88.7	8.7	6.8	11.3	31.2	25.1	--	4.9	.1	0
Pineapple	56.5	59.5	92.5	9.9	6.3	7.0	26.6	33.7	--	7.0	.6	0
Valencia	51.1	62.0	93.0	11.3	7.0	7.0	30.4	30.0	--	7.2	1.0	0
Tangerine												
Dancy	--	64.5	91.7	--	8.6	8.3	--	26.7	--	--	.2	0
Grapefruit												
Duncan	--	62.5	94.0	--	6.6	6.0	--	28.6	--	--	.3	0
Marsh	52.4	63.0	92.6	11.7	6.7	7.4	30.8	29.0	--	5.1	1.3	0

Source: (13, 15)

Table VII. Relative Amounts of Various Monosaccharides Found in the Hydrolysate of Polysaccharide Fraction of the Alcohol-Insoluble Solids of Citrus Peel, Pulp and Juice

Component part	Fractions	Arabinose	Xylose	Rhamnose	Galactose	Glucose	Galacturonic acid	Other uronic acids
Peel	Pectic substances	+	t	--	+	--	+++	--
	Hemicellulose	+	++	t	+	+	t	t
	Cellulose	+	+	--	t	+++	--	+
Pulp	Pectic substances	+	t	t	+	t	+++	--
	Hemicellulose	+	++	--	+	+	+	t
	Cellulose	+	+	t	+	+++	--	+
Juice	Pectic substances	+	t	t	+	--	+++	--
	Hemicellulose and							
	Cellulose	++	+	--	++	--	+	--

Source: (13, 17) + = < 25% of total polysaccharides

++ = between 25 and 50%

+++ = > 50%

t = trace

-- = not found

provide a good source of dietary fiber. Church and Church (22) reported that an average size orange could supply about 0.8 g of dietary fiber, whereas 236 ml (8 fl oz) serving of juice contains only one-half that amount.

Organic Acids. The most predominate soluble constituents of citrus juice, following the sugars, are the organic acids and their salts. They represent about 10 percent of the total soluble solids in citrus juices. The proper ratios of sugar and the acids and their buffers give the citrus juices their delightful taste.

The organic acids of citrus fruit include a group of carboxylic acids (23) with different acids predominant in various component parts of the fruit. Citric acid is the main acid in the juice, representing from 80 percent of the total acidity in juice from ripened oranges, about 90 percent of that of grapefruit and nearly all of that of lemon. The pH of the juice greatly affects the sourness of the product (24) and is, in turn, affected by the cations, especially potassium. The major acids in citrus peel are malic, oxalic (25), malonic (26), and quinic (27). Organic acids are metabolized in the body and should be considered as a source of energy. In the case of the salts of these acids, the organic portion is metabolized leaving the free cations to be combined with other anions. Thus, citrus juice is classed as an alkaline food (28).

Protein

The amount of protein in citrus fruit is relatively low (Table II), and the juice and peel have about the same amount (29). Much of the value that is considered as protein is either free amino acids or non-protein constituents which contain nitrogen. The total nitrogen of orange juices was found to increase with the maturity of the fruit and ranged between .068 to .120 g per 100 ml (30). The actual protein values obtained by Clements (31) were about 20 percent of the acetone powder. Nearly 30 percent of the alcohol-insoluble solids of juice and about 20 percent of that of vesicular pulp were found to be protein as determined by the Kjeldahl procedure (32). These values are the actual protein that was precipitated by alcohol and are only a fraction of the total protein values usually reported for orange juice (8). The main source of proteins in citrus juice is probably in the form of enzymes and the plastids. At least 47 different enzymes have been reported to occur in citrus fruits (33). Citrus fruits also contain several phenolic amines (34), some of which such as synepherine, may have physiological importance (35).

Among the various free amino acids reported in citrus juices (32), arginine is the only semi-indispensable amino acid that occurs in moderate amounts. The majority of amino acids in citrus are considered to be nonessential according to the classification

by Block and Bolling (36). At most, the contribution of free amino acids in citrus juices to human nutrition is minimal.

Lipids

From a dietary standpoint, the contribution of citrus lipids is insignificant; and only between .06 and .09 percent has been found in oranges (37). They are, however, of importance because of their effects on the development of off-flavors (12), thus lowering the palatability of these products. The near absence of lipids in citrus makes it a desirable food for those on a limited fat diet.

Micronutrients in Citrus

The term micro is used purely to indicate the physical quantities required in nutrition rather than for their importance. These nutrients represent the vitamins, minerals and other substances that have dietary significance.

Fat-Soluble Vitamins in Citrus Fruit

Among the several vitamins in this classification, only vitamin A is present in appreciable quantity as carotenoid provitamin A in citrus (38). No vitamin D has ever been reported in citrus nor any plant vitamin D precursors, such as ergosterol. Several of the sterols present in citrus fruits are reported (39, 40, 41), but they are not related to vitamin D.

Vitamin E. The amount of vitamin E in citrus is nutritionally insignificant. Braddock (42) reported only 0.1 mg in 100 ml of orange juice. The U.S. RDA for this vitamin is 30 mg. Newhall and Ting (43) found as much as 1 mg in 100 grams of flavedo on a fresh weight basis. Its antioxidant property plays an important role in the keeping quality of citrus oils.

Vitamin A. The vitamin A of citrus fruit is entirely in the form of provitamin A carotenoids. The carotenes (both the alpha and beta form) and the cryptoxanthins in citrus are considered as the main precursors. The carotenes are only a minor component of the total carotenoids of oranges ranging from about 5 to 10 percent (44-46). In Dancy tangerine and Valencia oranges, cryptoxanthin is the main vitamin A precursor (47, 48). High performance liquid chromatography (HPLC) has been used to separate cryptoxanthin from other oxygenated carotenoids (49, 50). Only the beta-cryptoxanthin has provitamin activity. Prior use of open column chromatography (51) could not separate the different mono-oxygenated carotenoids, thus giving higher values. Using the HPLC method, Stewart (52) analyzed the carotenoids of several varieties of oranges and mandarins, and found that beta-cryptoxanthin is the

main provitamin A citrus carotenoid. The mandarin-type citrus fruit is a good source of provitamin A, whereas oranges are significantly poorer (Table VIII). With grapefruit, the white varieties have no vitamin A, but the pink and red varieties have been found to contain appreciable amounts of beta-carotene (53, 54).

Table VIII. Vitamin A Content of Citrus Juices

Fruit	% U. S. RDA 177 ml (6 fl. oz)
<u>Oranges</u>	
Early and mid-season	1.6-2.7
Late season	1.7
<u>Tangerines</u>	
Dancy	19
Honey	64
Robinson	23
<u>Tangelos</u>	
Orlando	4.7
<u>Grapefruit</u>	
White	None
Ruby	30

Source: (44, 52-54)

Water-Soluble Vitamins in Citrus Fruit

Vitamin C. Perhaps the most important contribution of citrus fruits to human nutrition is attributed to their high ascorbic acid. Although citrus products are not the only source for high contents of vitamin C among fruits and vegetables, their popularity are largely due to their desirable flavor, taste and color. In Table IX (55) are listed some of the more common fruits and vegetables and their vitamin C content. An average 177 ml (6 fl oz) serving of either orange or grapefruit juice could provide 100 percent U.S. RDA of vitamin C (60 mg). Tangerine juice, although containing less vitamin C than other citrus juices, would provide a substantial amount toward the recommended daily allowance.

Nearly 3/4 of all vitamin C in an orange and 5/6 in a grapefruit is found in the peel (56), however, citrus juices and their products provide a major portion of the vitamin C in the American diet. Considerable variations in vitamin C content can be found in different citrus products due to such factors as varieties, maturity and cultural practices of the fruit (57) from which the products originate and to the processing practices and storage conditions of these products before they reach the consumer.

The decrease of ascorbic acid with fruit maturity is illus-

Table IX. Average Vitamin C Content of Freshly Harvested Fruits and Vegetables

<u>Product</u>	<u>mg/100 g</u>
Guava	300
Broccoli	120
Green pepper	120
Turnip greens	120
Cabbage	60
Orange	50
Lemon	50
Grapefruit	40
Tangerine	25
Potato	30
Tomato	25
Pineapple	25
Banana	10
Apple	10
Peach	4

Source: (55)

trated in Figure 1. Vitamin C content of the early and midseason oranges are higher than that of the late season fruit. These facts indicate that high quality does not correlate with higher nutritional quality. The more mature fruit and those of the late season variety are generally regarded as of better quality.

Stability of Vitamin C in Citrus Fruits and Juices

The ease of oxidation of reduced ascorbic acid is the basis for a simple method of analysis by dye titration (58). Ascorbic acid as it occurs in citrus juice is in the reduced form. When subjected to oxidation, ascorbic acid changes to the dehydro form. Dehydroascorbic acid has nearly the same physiological activity as the reduced form and is easily converted to the latter. Further oxidation of the dehydroascorbic acid converts it to 2,3-diketogulonic acid. This reaction is irreversible, and the oxidized product is devoid of biological activity. These reactions are shown in Figure 2. Nearly 90 percent or more of the vitamin C found in citrus juice and citrus products is in the reduced form (Table X) (59).

Vitamin C in citrus juice is remarkably stable during the short period it is generally kept after extracted from the fruit. Freshly extracted orange and grapefruit juices retained about 98 percent of their original vitamin C at 21.1° for 3 days. At 4.4° orange and grapefruit juices retained 96 and 99 percent, respectively, of the original amount after one-week's storage (60). Orange juice that has been heated to boiling for 15 min still retained about 96 percent of the vitamin C (59). Atmospheric oxygen

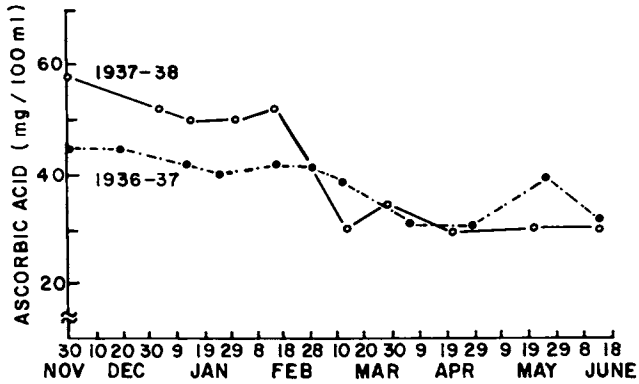


Figure 1. Seasonal changes of ascorbic acid in juice of Florida oranges (average values for 2 seasons)

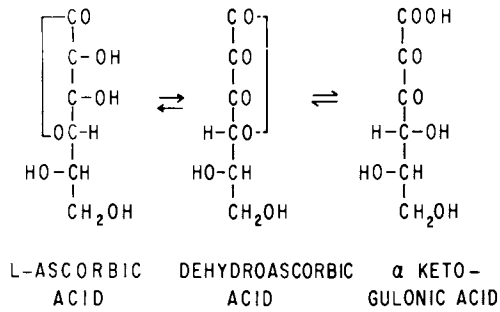


Figure 2. Oxidation of L-ascorbic acid

Table X. Reduced Ascorbic Acid and Dehydroascorbic Acid in Citrus Products

<u>Product</u>	<u>Reduced</u> <u>(mg/100 g)</u>	<u>Dehydro</u>
Canned orange juice	43.1	0.9
grapefruit juice	32.0	1.8
tangerine juice	27.2	1.1
FCOJ	45.2	1.6
Orange segments	36.0	1.5
Grapefruit segments	31.5	2.3

Source: (59)

is an essential element in the loss of vitamin C during long term storage. In sealed containers, there is a rapid loss of the vitamin followed by a slow decrease and becoming relatively constant. Smoot and Nagy (61) found that when stored at high temperature, the loss of vitamin C continues even after the headspace oxygen is exhausted and that the diketogulonic acid fraction increased noticeably.

A comparison of the vitamin C content of freshly squeezed orange juice and reconstituted FCOJ showed no marked difference in the ascorbic acid content during storage in a home refrigerator for up to one week (62) (Figure 3). The loss of vitamin C in intact oranges during their marketing period are not expected to be more than 10 percent of the original (63).

Thiamine (Vitamin B₁). Citrus products are also good sources of thiamine. They provide comparable amount or more of vitamin B₁ than foods that are known suppliers of this nutrient on the basis of nutrient density as measured by the Index of Nutrient Quality (INQ) (64). Listed in Table XI are the INQ of some common foods. The vegetables have high INQ since the index is calculated on unit nutrient per KCal, but citrus products are higher in INQ than some other fruits. Wholewheat bread, usually considered as a source of vitamin B₁, has an INQ much lower than that of orange juice. Thiamine in canned orange juice is rather stable. A loss of only 16-17 percent was reported after the product was stored for 18 months at 27° (65).

Folic Acid (folate). Chemically, folic acid is a pteryl-glutamic acid. The several forms that occur in nature depend on the numbers of glutamic acid units and methyl groups in the molecules. Because of its usual low concentration, folic acid is generally determined in food materials by the microbiological assay with *Lactobacillus casei* and measured turbidimetrically or titrimetrically. Deficiency of this vitamin could result in macrocytic anemia (66). Orange juice contains more folate than many other fruit juice (Table XII)(67).

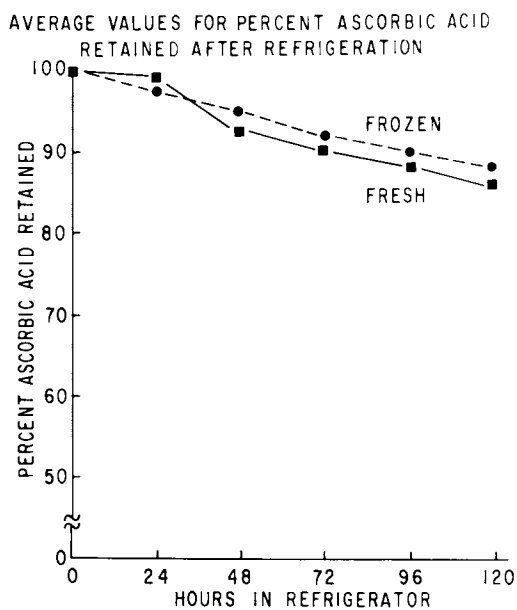


Figure 3. Changes in ascorbic acid content of freshly squeezed orange juice and reconstituted orange juice concentrate during storage in refrigerator (62)

Table XI. Comparison of Index of Nutrient Quality (INQ) of the Amino Acids of Citrus Products With Those of Various Other Common Foods

Foods	Index of nutrient quality (INQ)
Milk (whole)	1.20
Milk (skim)	2.12
Eggs (hard-boiled)	1.00
Beef	.33
Grapes (seedless)	1.71
Grape juice	.74
Apple (whole)	1.00
Apple juice	.33
Peaches (raw)	1.00
Peaches (canned in water)	.53
Banana	1.20
Grapefruit segments	2.00
Grapefruit juice (canned)	1.40
Grapefruit juice (from concentrate)	2.00
Orange (peeled)	4.00
Orange juice (canned)	2.83
Orange juice (from concentrate)	3.83
Tangerine (peeled)	2.50
Tangerine juice (canned)	2.40
Broccoli (cooked)	4.00
Cabbage (raw)	5.00
Tomato (raw)	5.60
Tomato juice	5.33
White bread	2.94
Whole wheat bread	2.50

Source: (64)

Table XII. Folic Acid Content of Some Fruit Juices

Product	Average	Range (micrograms/100 ml)
Orange juice	35	26-40
Mixed fruit juices (infant)	8	7-10
Grapefruit juice	8	3-24
Tangerine juice	21	17-25
Grape juice	0.1	0.1
Prune juice	0.3	0.2-0.3
Apple juice	0.2	0.1-0.2
Tomato juice	9.9	9.7-10

Source: (67)

Vitamin B₆. Analysis of reconstituted frozen concentrated orange juice has shown that it contains an average of 55 mcg per 100 ml (68). While this amount is not considered high, orange juice is comparable to milk in supplying this nutrient. Atkin *et al.* (69) reported that cow's milk supplies an average of 54 mcg of vitamin B₆ per 100 ml and orange juice 56 mcg in equal volume. Canned and fresh grapefruit juices contain 8 and 18 mcg per 100 ml, respectively (70). Vitamin B₆ is a group of 3 related compounds, namely pyridoxine, pyridoxamine and pyridoxal. It is essential for many of the enzymes in amino acid metabolism. Determinations of vitamin B₆ in citrus are made by microbiological assay. Although the RDA for this nutrient is 2 mg, the actual requirement could be as low as 1.25 mg. With high protein intake, the need could be as much as 1.75 mg (71).

Niacin, Riboflavin and Pantothenic Acid. These three vitamins are all present in citrus juices but the amount of each present in orange juice is only between 2 to 4 percent U.S. RDA in a 177 ml serving (68). In grapefruit juice, the amount is slightly less but is also near the 2 percent U.S. RDA region (72). While these amounts are not large, they are, however, greater in proportion to the average caloric intake as expressed by the INQ (7).

Mineral Nutrients in Citrus

Potassium and Sodium. Potassium is the most abundant mineral of citrus juices and other citrus products, amounting to 40 percent of the total ash (73). In contrast, citrus fruits are low in sodium, generally less than 1 mg/100 ml juice (74). In 100 ml of orange juice, 4 to 6 meq of potassium may be available (73). Slightly less was found in canned grapefruit juice (72).

These two elements are the main cations of the cell. Persons with high blood pressures are usually placed on a low sodium diet. Although potassium deficiency in normal adults is rare,

people on diuretic medicine or on an improper diet have been known to need a supplemented intake of this element.

Calcium, Magnesium and Phosphorus. Calcium and magnesium are the two major divalent cations of citrus fruit, but both occur in relatively low amounts in the juices ranging between 6-15 mg/100 ml (74). The contribution of citrus juices for these two mineral nutrients would only be 2-3 percent U.S. RDA per a serving of 177 ml (6 fl oz). However, when calculated on the basis of caloric intake, citrus juices provide all these three nutrients (phosphorus, calcium and magnesium) near or above their caloric contributions.

While phosphorus is related to the two bivalent cation in human nutrition as in structural formation, it is also present in the blood cells as phosphates and in protein, lipids, carbohydrates and enzymes such as ATP and ADP. The U.S. RDA for phosphorus is one gram, and orange juice and grapefruit juice may contain between 15 to 20 mg per 100 ml.

Trace Elements as Mineral Nutrients. Within this group of elements are copper, zinc, iron and manganese. All of these minerals are supplied during the cultivation of citrus fruit, and are essential as plant nutrients. They are important in many of the enzymatic reactions in the metabolic activities of the body. These minerals are all found to be near, or slightly above, the caloric contribution of the citrus products (7, 75).

Other Constituents in Citrus with Possible Dietary Importance

Several constituents of citrus fruits have been reported to have dietary importance, although there are strong controversies about them. While these substances do not cause deficiency symptoms when not taken in sufficient quantities, their presence in food may contribute to the betterment of health. Among them are the bioflavonoids and inositol.

Bioflavonoids

Flavonoids are a group of compounds containing a $C_6-C_3-C_6$ structure. At least 50 flavonoids have been isolated and identified from citrus (76), but only two are found in appreciable quantities. Hesperidin is the major flavonoid in oranges whereas naringin is major in grapefruit. Some flavonoids have been found to have biological activities; the term bioflavonoids is sometimes used to describe these substances. Szent-Gyorgyi (77) had considered calling these substances "Vitamin P". Since these substances are not essential in the human diet, in as much as their deficiency does not cause specific symptoms, the term "Vitamin P" was not accepted by most nutritionists. Tests of some of the bioflavonoid preparations failed to show any physiological

activities. Thus, it was concluded by many nutritionists that the bioflavonoids are not of any nutritional importance. Bioflavonoids as isolated from oranges for pharmaceutical purposes contain mostly hesperidin, and when purified, only hesperidin may be present. The physiological activities, however, are present in some of the minor components of the citrus flavonoid pool. Robbins (78) found that the fully methoxylated flavones, especially nobiletin and tangeretin, and heptamethoxyflavone are effective in preventing the adhesion of red blood cells. The decrease in this red blood cell adhesion has been associated with a lessening of some heart diseases.

Inositol

Inositol is a commonly occurring substance in plants and has been found to be present in citrus juice in the amount of about 150-200 mg/100 ml (79). It has been considered a growth factor for some animals (80) but it has not been proven essential in humans. It is doubtful that this substance is critical in the human diet.

Conclusion

With increasing knowledge of nutrition and of nutrients and food values of citrus fruit and with the development of technology of processing, packaging, and transportation to reduce the cost of the product, citrus fruits and their products have become staple food items in most developed and some developing countries in recent years. The superiority of citrus is not only dependent on the fact that it contains high vitamin C, although it is important, but that it contains several hundred other chemical constituents including many other vitamins, polysaccharides, amino acids and minerals. Many of these are essential in human nutrition. The inclusion of citrus in the diet provides sugars as a quick source for energy as well as many other substances which may contribute to a person's well being.

The major assets of citrus as food are their desirable characteristic aroma, taste and color and in the interrelationship of all the dietary nutrients in a pool as in all natural foods such as citrus when consumed. As a food it can be enjoyed by persons of all ages and at all times. Many citrus constituents either already identified or to be isolated may contribute to the betterment of health.

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Studies on the Role of Citrus in Health and Disease

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Since 1973, we at the University of Florida have been systematically evaluating the role of citrus fruits and their products in human nutrition. Our previous studies of citrus have yielded valuable information relating to the beneficial nutritional role of citrus in providing certain vitamins, replenishing lost electrolytes, and possibly inhibiting viral infections. Our ongoing research in the area of study of the chemistry and biological role of dietary citrus pectin in human nutrition has yielded results elucidating the complex chemical nature of pectin and the possible biochemical basis by which dietary pectin may cause lowering of serum and/or liver cholesterol. In addition, another series of ongoing experiments suggest that citrus pectin may influence the biological processes regulating the absorption of glucose in patients with postprandial hypoglycemia.

This article is a review of our major biomedical research efforts, previous and ongoing, on the role of citrus and citrus pectin in human nutrition. Its purpose is to focus attention, provide scientific evidence and evaluate the direction of future research on the beneficial role of citrus fruits in human nutrition.

NUTRITIONAL ROLE OF CITRUS BEVERAGES

Bioavailability of Water-soluble Vitamins

Nutritionally, the most important water-soluble vitamins in citrus fruits are ascorbic acid, folic acid and pyridoxine. Clinical studies on the bioavailability of these vitamins, as well as basic research on the absorption and chemistry of these vitamins, have yielded valuable information adding to our overall understanding of the nutritional quality and bioavailability of these vitamins found in citrus fruits.

Ascorbic Acid. Unlike a number of animal species, man and

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other primates as well as the guinea pig depend on dietary sources for their vitamin C needs because these species lack the biochemical mechanism to synthesize this essential nutrient. Lack of vitamin C in dietary sources causes scurvy in adults which is characterized by sore spongy gums, impaired capillary integrity with subcutaneous hemorrhages and edema, joint pain, anorexia and anemia. In children, vitamin C deficiency causes tenderness and swelling of joints, arrested skeletal development, impaired wound healing, anemia and inadequate tooth development.

Citrus juice is rich in ascorbic acid and, therefore, is an important dietary source of this essential vitamin. Our studies were aimed at defining whether vitamin C from citrus sources is comparable with the synthetic vitamin in terms of bioavailability and intestinal absorption. These studies were conducted using human volunteers and guinea pig experimental models (1,2).

Prior to our work, absorption of vitamin C from intestine was believed to be passive, i.e., not involving active transport. Contrary to this belief, results from our *in vivo* perfusion studies on the saturation kinetics relating to the intestinal absorption of pharmacological doses of ascorbic acid in human volunteers and guinea pigs demonstrated that intestinal absorption of ascorbic acid is accomplished by an active transport mechanism, supporting the observations made previously by Stevenson (3). The observed active transport of ascorbic acid showed parameters characteristic of the phenomenon of saturability, i.e., as the dose of vitamin C presented to the intestine is increased, the relative proportion of ascorbic acid absorbed showed a decrease. The most efficient absorption of the vitamin was observed when amounts nearly identical to that present in orange juice were presented to the intestine. These observations were further corroborated by studies on the absorption of vitamin C *in vitro* on isolated jejunum and ileum segments of guinea pig with intact vascular supply using experimental guinea pig models. These studies, in addition to establishment of the active absorption of vitamin C by intestine, provided conclusive evidence that human volunteers perfused with pharmacological doses of vitamin C absorbed the vitamin less efficiently. Furthermore, it was shown that the amount of vitamin C present in orange juice is near optimum in terms of its efficient absorption by intestine, and the pharmacological doses of vitamin C supplied to human volunteers would appear to be of little or no additional nutritional value and may even be detrimental.

Folic Acid. Citrus juice both in frozen or fresh form is a rich and stable source of folate. The presence of vitamin C in orange juice protects it from oxidation and, unlike other nutritional sources of folate, the folate in orange juice is not subjected to destruction caused by cooking of foodstuff.

Research conducted in our laboratories has clearly established that, in addition to being rich in folate, citrus juice

contains N-5 methyl tetrafolate as the most predominant chemical form, which is metabolically active and most readily available. Its natural occurrence is unique in citrus fruits (4-6). Furthermore, by employing triple lumen perfusion techniques, it was shown that diphenylhydantoin does not influence the absorption of citrus folate in human volunteers. This may be clinically significant, since diphenylhydantoin in dosages sufficient to control epileptic seizures influences absorption of forms of folate present in other food sources.

Vitamin B-6. Comparative studies on the bioavailability of synthetic and naturally occurring forms of vitamin B-6 found in orange juice have demonstrated that the absorption of vitamin B-6 from orange juice in human volunteers is not efficient when compared with the synthetic forms of this vitamin (7). These observations are similar to the findings of others (8) that natural nutritional sources contain the B-6 largely in bound form and cooking of foodstuff enhances the bioavailability of this vitamin in animals. Naturally occurring vitamin B-6 is generally bound to proteins (9), however citrus fruit, which contains substantial amounts of bioavailable forms of vitamin B-6, is low in protein contents. Further investigations on the nature of the chemical component bound to vitamin B-6 found in citrus fruit have demonstrated that in citrus fruit vitamin B-6 is bound to a small (molecular weight: <3,500) heat-stable and non-proteinic molecule which exhibits binding to both pyridoxal and pyridoxine (10). These studies have contributed to our basic understanding of the bioavailability, absorption and chemistry of vitamin B-6 in general.

Electrolyte Balance

It has been previously demonstrated that considerable quantities of sodium and potassium are lost through sweating (11,12). Loss of potassium from skin has been estimated to be of the magnitude of 44% of the total potassium losses. Furthermore, loss of potassium caused by strenuous physical activity in hot climates may cause rhabdomyolysis and myoglobinuria.

Studies (13,14) were carried out to evaluate the role of an orange juice based "thirst-quencher" in replenishing the lost electrolyte balance caused by extreme exercise in a hot and humid climate, since orange juice thirst-quencher contains appreciable amounts of sodium and potassium. Members of the University of Florida Track Club were selected and studied for the sodium and potassium electrolyte balance changes caused by exercise, and monitored by employing whole body counting techniques using ⁴⁰K. These athletes ran 18 to 20 miles a day and were given three individual liquid electrolyte supplements, namely: Gatorade^R, orange juice thirst-quencher and an electrolyte-sodium only solution. Results obtained from these studies demonstrated that only the

orange juice thirst-quencher with its high potassium content resulted in a positive potassium balance which even exceeded requirements recommended by the National Research Council for athletes. These findings lend additional support to the nutritive value of citrus beverages in maintaining the electrolyte balance in exercise, as well as in overall human health since potassium is essential for normal function of muscles, including the heart.

Resistance to Viral Infections

Although highly controversial, evidence has been presented in the literature which suggests that vitamin C could be involved in increasing tissue resistance to respiratory viruses (15). Orange juice is a natural source of vitamin C containing an average of 30 mg of vitamin C per 100 ml of juice. Studies (16) were carried out on the effect of ingestion of orange juice on Rubella virus infections in human volunteers who were infected with the virus either by nasal instillation or subcutaneous injection. Using this model, human volunteers infected intranasally develop respiratory symptoms whereas subcutaneous introduction of virus leads to development of systemic symptoms. The infected individuals were given a liter of orange juice a day whereas matched normal controls were instructed to eliminate citrus from their daily diet and not to take vitamin supplements. Results obtained from these preliminary experiments suggested that ingestion of orange juice might have an inhibitory influence on the development of only respiratory tract symptoms since no such influence on the development of systemic symptoms was observed in human volunteers infected subcutaneously. In addition, serum antibodies to Rubella virus appeared significantly earlier in individuals infected through the nose.

NUTRITIONAL ROLE OF DIETARY CITRUS PECTIN

Diseases such as atherosclerosis, colon cancer, constipation, gallstones and many other so-called "ailments of the Western world" are linked to a common etiology, i.e., deficiency of fiber in the diet. Atherosclerosis and coronary artery disease occur more frequently in populations showing a high incidence of hypercholesterolemia than in those showing hypocholesterolemia. Cholesterol is the major component of the atherosclerotic lesions which are characterized by intimal proliferation of smooth muscle cells accompanied by an accumulation of large amounts of connective tissue components such as collagen, elastin, glycosaminoglycans and deposition of extra- and intracellular lipid.

Elevated serum cholesterol levels are invariably associated with the etiology of atherosclerosis and coronary artery disease, and it has been shown by several investigators that pectin from a variety of sources when supplemented in the diet of a number of laboratory animals, as well as human volunteers, causes lowering

of liver and/or serum cholesterol levels (17). Furthermore, pectin has been shown to retard induced avian atherosclerosis (18).

Does Citrus Pectin Bind Bile Salts? A possible mechanism by which dietary pectin may cause lowering of cholesterol levels in rats has been reported (19). In these in vitro studies, pectin was found to inhibit the transport of taurocholic acid from everted sacs of rat intestine. The absorption of labelled cholesterol was depressed by the addition of 5% pectin to the diet as evidenced by increased excretion of labelled cholesterol and diminished cholesterol deposition in the liver. It was concluded from these studies that pectin lowers cholesterol levels in cholesterol-fed rats primarily by binding bile salts and, consequently, by impairing cholesterol absorption. Results similar to those obtained with dietary pectin and described have also been reported for other non-nutritive substances such as guar gum, psyllium seed colloid and seruglucan (20).

Dietary fibers in general have traditionally been tested for their binding of bile salts by employing centrifugation techniques. These analytical methods are not applicable to water-soluble mucilaginous fibers such as pectin. Therefore, in our attempts to extend and demonstrate similar binding of bile salts to citrus pectin, we have developed a methodology based on equilibrium dialysis to study interaction of bile salts with fibrous as well as mucilaginous dietary fiber. This method has been successfully utilized by us to study and compare fiber-bile salt binding and/or interaction for a variety of fibers, including pectin. In our studies we compared and studied the binding of bile salts by a variety of nonnutritive fibers by using both centrifugation and equilibrium dialysis techniques. These studies were carried out by utilizing ^{14}C sodium taurocholate. Centrifugation studies were performed according to established techniques. Equilibrium dialysis studies were performed utilizing Spectrapor 2 membrane and a Spectrum dialyzer. The percentage of binding was determined by assaying the radioactivity in the supernatant fluid obtained following centrifugation and in the dialysate solution opposite to the fiber-containing half cell. Comparable results were obtained by the two methods used to study the binding of bile salts, with an incubation mixture containing 2.5 mM of sodium taurocholate per mg of fiber. Bile salts binding was compared by these two methods using cholestyramine, a known resin which binds bile salts, ground alfalfa, miller's bran, cellulose, and citrus pectin and lignin. As summarized in Table I, results obtained from these studies clearly demonstrated the binding of alfalfa (8.4% by centrifugation vs. 14.4% by equilibrium), bran (1.7% by centrifugation vs. 8.5% by equilibrium) and lignin (2.4% by centrifugation vs. 1.4% by equilibrium) to sodium taurocholate. Binding of taurocholic acid to cholestyramine was found to be identical (84%) by the two methods. However, no binding of bile salts to pectin or cellulose was observed. Results obtained from these studies

Table I. In vitro interaction of bile salts with pectin and other dietary fibers

	Equilibrium dialysis (% bound)	Centrifugation (% bound)
alfalfa	14.4	8.4
bran	8.5	1.7
cellulose	nil	nil
pectin	nil	technical difficulties
lignin	1.4	2.4
cholestyramine	84.0	84.0

suggest that bran and alfalfa, although clinically less effective in lowering of serum and/or liver cholesterol levels, did bind small amounts of bile salt but only a fraction of that bound by cholestyramine resin, while pectin which is unique in causing lowering of cholesterol levels demonstrated no binding to bile salts. Although care must be taken in extrapolating these findings to *in vivo* processes which remain undefined, it was concluded that the phenomenon of binding of bile salts to dietary fiber, particularly pectin, is not sufficient to explain the biochemical basis by which dietary pectin causes lowering of cholesterol levels. It was, therefore, imperative to search for alternate biochemical and physiologic mechanisms to help explain the hypocholesterolemic effect of dietary pectin.

Interaction of Pectin with Serum Lipoproteins. Earlier in the course of epidemiologic studies showing a strong relationship between elevated levels of serum cholesterol and subsequent development of atherosclerosis, it was discovered that cholesterol found in plasma did not occur in the free state but was bound/carried in various lipoprotein fractions. Lipoproteins found in plasma are lipid-protein complexes of various sizes and densities. These complexes are constructed with the charged protein molecules on the surface and the nonpolar molecules, such as triglycerides and esters of cholesterol, on the inside. The lipoproteins function to transport cholesterol in a water-soluble form. Based on their densities, sizes and behavior upon preparative ultracentrifugation, four major kinds of lipoproteins are characterized to date. These are: chylomicrons which carry dietary triglycerides from intestine to nonhepatic tissues for utilization or storage, very low density lipoproteins (VLDL) containing triglycerides made primarily in the liver, the low density lipoproteins (LDL) and the high density lipoproteins (HDL). Of these lipoproteins, LDL are the major carriers of circulating cholesterol. Elevated levels of LDL in the serum contribute significantly to the coronary heart disease risk in persons older than age 50. On the other hand, elevated levels of HDL, which carry approximately one quarter of the cholesterol found in serum, has a beneficial influence on the overall health of individuals. In fact, increased levels of HDL in serum has been attributed with longevity (21). The role of VLDL in causing atherosclerosis remains unclear. It, therefore, follows that much of what has been learned in the past about the ill effects of high serum cholesterol can be attributed to the associated elevated levels of LDL or cholesterol carried in this lipoprotein fraction. Furthermore, results from recent studies (22) have shown that LDL, which are known to carry most of the cholesterol found in blood, play a key role in both the development of atherosclerotic lesions and in the regulation of cholesterol metabolism in a variety of cells. These studies suggest that specific binding sites for LDL present in normal cells are absent and/or defective in fibroblasts from subjects

with homozygous familial hypercholesterolemia. Interaction of LDL with a specific receptor at the cell surface of normal cells initiates a series of still poorly understood complex processes leading to endocytosis of LDL, the lysosomal degradation of internalized LDL and suppression of cholesterol synthesis in the cell. In individuals homozygous to familial hypercholesterolemia, a lack of binding sites at the surface of fibroblasts from these individuals lead to accumulation of large quantities of LDL in the blood stream and the development of severe, atherosclerotic disease. Results from these studies clearly suggest that serum cholesterol, most of which is carried in LDL, is causally involved in atherogenesis.

Pectin, a polyanionic heterogeneous mixture of complex polysaccharides of high molecular weights, is predominantly composed of linear (1-5) linked galactopyranosyl uronic acid residues (23). Like pectic polysaccharides, glycosaminoglycans are also polyanionic polysaccharides containing alternating uronic acid (L-iduronic acid and/or D-glucuronic acid) and hexosamine (D-glucosamine or D-galactosamine) residues and, exclusive of hyaluronic acid, all glycosaminoglycans are sulphated. In atherosclerosis, the interaction of glycosaminoglycans with lipoproteins has been suggested as being involved in the mechanism of sequestering LDL at the endothelial surface (24). Evidence for complex formation between LDL and glycosaminoglycans in human aorta intimal layer and demonstration of a correlation between the severity of atherosclerosis and the amount of LDL present in intima has been presented by several investigators (25). This interaction between polyanionic glycosaminoglycans and the cationic protein moiety of lipoproteins appears to be dependent upon the electrostatic forces between the two macromolecules (26).

Based on our knowledge of the facts that (a) lipoproteins are carriers of cholesterol in the blood stream, (b) they are involved in atherogenesis, (c) pectin when supplemented in diet causes lowering of serum and/or liver cholesterol in man as well as a number of laboratory animals and (d) polyanionic glycosaminoglycans interact with lipoproteins, it was of interest to us to investigate the interaction of polyanionic pectin with lipoproteins in order to explain the biochemical basis by which pectin may cause lowering of serum/liver cholesterol levels.

To test this hypothesis, very low density lipoprotein (VLDL, $d < 1.0$ gm/ml), low density lipoprotein (LDL, $d = 1.02-1.063$) and high density lipoprotein (HDL, $d = 1.09-1.21$) were isolated from outdated human plasma by ultracentrifugation according to established procedures (27,28), using potassium bromide for density adjustments and stored at -20° C in the presence of 20% sucrose before use. The purity of individual lipoprotein fractions thus obtained was established by polyacrylamide gel electrophoresis in sodium dodecyl buffer system (29) and filtration through a Sepharose 6B column, equilibrated with 0.2 M potassium bromide in 0.1 M sodium phosphate buffer, pH 7.2. Protein (30) and cholesterol

contents of various lipoprotein fractions were estimated according to established procedures.

Commercially available grapefruit (*Citrus paradisi*) pectin, a gift from Lykes-Pasco Packing Company, Dade City FL, was reprecipitated three times using 70% ethanol prior to study of the interaction of pectin with serum lipoproteins. In addition, pectin from grapefruit albedo was also extracted in our laboratory according to established procedures of Thornber and Northcote (32). The chemical composition of the two pectins was found to be similar. These pectin preparations contained galacturonic acid which accounted for 76-78% by weight of the total pectin. The remainder was accounted for by neutral sugar components, primarily galactose and arabinose and trace amounts of rhamnose, xylose, mannose and glucose.

Interaction of pectin with various lipoprotein fractions was studied according to established analytical techniques successfully utilized to study interaction of glycosaminoglycans with serum lipoproteins as revealed by the formation of insoluble complexes (33). Pectin and lipoprotein preparations were dialysed against appropriate buffer solutions and the interaction was studied over a wide range of pectin:lipoprotein ratio, pH and a variety of experimental conditions. To test tubes, each containing 0.5 mg of lipoprotein in 0.2 ml of buffer, was added variable amounts of pectin solutions and the final volume of reaction mixture was brought to 4.0 ml with appropriate buffer. After incubation of reaction mixture for 15 min at room temperature, the formation of insoluble complexes was assayed by recording absorbance at 680 nm. Test tubes containing buffer and pectin, or lipoprotein alone, were also run in parallel, to serve as controls.

Results obtained from these studies revealed that, of all the lipoprotein fractions tested, the formation of insoluble complexes with pectin was limited specifically to LDL. The pH optimum of the observed interaction was found between pH 4.3 and 5.2. The interaction between pectin and LDL was found to be optimal in 0.05 M phosphate buffer, and increase in the molarity of buffer inhibited the formation of complexes. Furthermore, presence of divalent cations such as Mg, Ca and Mn in concentrations above 1 mM in the reaction mixture caused the inhibition of formation of complexes between pectin and LDL.

Although several investigators have previously demonstrated the *in vitro* interaction of serum lipoproteins with glycosaminoglycans, to our knowledge the interaction between serum lipoproteins and dietary pectin has never before been investigated. The results obtained from the *in vitro* studies described here clearly suggest that pectin interacts specifically with serum LDL and this interaction appears to be electrostatic in nature. In addition, the observed interaction appears to be of potential significance since LDL is the major biological carrier of cholesterol and the principal ingredient of atherosclerotic lesions found in diseased cardiovascular tissue. The observed interaction assumes

additional significance when one considers our unsuccessful attempts described earlier to demonstrate the binding of bile salts to pectin as the only suggested biochemical basis by which dietary pectin may cause the lowering of cholesterol levels.

The conclusion reached in our laboratory that pectin interacts primarily with LDL, although of significance, is at best only preliminary. A number of questions remain unanswered. These include (a) Is the *in vitro* interaction between LDL and pectin of any physiological importance? (b) Is there a formation of soluble complexes between LDL and pectin under physiological conditions? (c) Of a number of polysaccharides found in pectin, which unique polysaccharide is primarily involved in the observed interaction? and (d) Could this observed interaction have the merit to explain the biochemical basis by which dietary pectin may cause lowering of serum cholesterol levels? A prerequisite to answering these questions is the availability of individual and highly pure and structurally defined polysaccharides found in citrus pectin. In addition, availability of radiolabelled pectic polysaccharides would greatly enhance our understanding of the problems and questions posed earlier. We have been able to achieve fractionation as well as labelling of several polysaccharides found in citrus pectin. These studies are described as follows.

Fractionation and Chemistry of Citrus Pectic Polysaccharides.

Pectic polysaccharides, commonly known as pectin, appear early in plant cell-wall formation. A series of complex biochemical steps results in the formation of cell plates followed first by its growth in area (primary cell wall) then in thickness (secondary cell wall). Exclusive of randomly oriented cellulose fibrils, primary cell wall is composed mainly of pectic polysaccharides (34). These pectic polysaccharides are rich in D-galacturonic acid, D-galactose and L-arabinose residues. With growth in thickness of cell wall (secondary cell wall), there appears to be a replacement of pectic polysaccharide deposition with polysaccharides rich in D-glucuronic acid or 4-O-methyl-D-glucuronic acid, D-xylose and D-glucose rich polysaccharides.

Earlier studies (35) led to the belief that pectic polysaccharides were a complex mixture of three groups of polysaccharides, pectic acid containing chains of 1-4 linked D-galacturonic acid, a galactan containing chains of 1-4 linked β -D-galactopyranose residues, and a highly branched araban containing 1-5 and 1-3 linked L-arabofuranose residues. Later studies (36) on the chemistry of pectic polysaccharides found in sisal plant pointed out that pectic acid-like substances are not polymers of D-galacturonic acid alone. Neutral sugars, especially D-galactose, L-arabinose and L-rhamnose, are integral components of acidic polysaccharides and linked to galacturonic acid chains. Further studies (37) on the structure and analysis of pectic polysaccharides from Lucerne have shown that the main chain of pectic acid

polysaccharides was composed of 1-4 linked D-galacturonic acid residues. However, the isolation of a partial hydrolysis product, the aldobiuronic acid, 2-O-(β -D-galacturonic acid) L-rhamnose provided evidence that the L-rhamnose residues were linked glycosidically to D-galacturonic acid and that L-rhamnose residues may act as branching points. Studies on the structure and analysis of pectic polysaccharides from soybean cotyledon meal (38) and lemon peel pectin (23) revealed that L-arabinose and D-galactose residues were also integrated in the framework of D-galacturonic acid and the chain of 1-4 linked D-galacturonic acid was interrupted frequently by L-rhamnose residues. Traces of 2-O-methyl-D-xylose and L-fucose were also found in the pectic polysaccharides obtained from these tissues. In addition to these neutral sugar components found in pectic polysaccharides described above, D-apiiose, a natural 5-carbon branched sugar, is also found in the pectic polysaccharides found in plants of the family, *Zosteraceae* (39). Furthermore, the cell wall polysaccharides from a number of plant sources appear to be covalently linked to a hydroxy-proline rich protein, extensin (40), adding further additional complexity to the chemistry of cell wall associated pectic polysaccharides.

Classic methods in use to fractionate plant cell wall polysaccharides are mainly based upon the differential solubilities of various cell wall polysaccharide constituents (32). To solubilize pectic polysaccharides, the cell wall material is extracted with water, ammonium oxalate or disodium EDTA. The solubilization of pectic polysaccharides could then be followed by solubilization of hemicelluloses, i.e., polysaccharides rich in D-glucuronic acid 4-O-methyl-D-glucuronic acid, D-xylose and D-glucose residues. The solubilization of hemicellulosic polysaccharides is accomplished by treatment of water, ammonium oxalate or EDTA insoluble material with a strong alkali.

We have recently achieved extraction of pectic polysaccharides from grapefruit primarily based on a procedure described by Thornber and Northcote (26). Grapefruit albedo was cut into small pieces and homogenized using a Vitris 45 homogenizer in hot 80% (v/v) ethanol. The ethanol-soluble material was removed from the insoluble residue by centrifugation of homogenate at 2000 x g for 30 min. This extraction with ethanol was repeated three times, following which the insoluble residue was recovered and extracted by continuous shaking in 2 volumes of chloroform:methanol (1:1 v/v) for 2 days at room temperature. At every 24 h interval chloroform:methanol soluble material was removed by centrifugation and fresh chloroform:methanol was added to continue extraction. The resulting delipidated, depigmented and dehydrated cell wall fraction was then air-dried and weighed. Pectic polysaccharides were then extracted from this dried residue with continuous shaking in 0.2 M disodium EDTA for 24 h and then solubilized pectic polysaccharides were removed by centrifugation. This extraction procedure was repeated and the EDTA soluble fractions thus

obtained were pooled and dialyzed extensively against running de-ionized distilled water. To the dialyzed EDTA soluble fraction representing pectic polysaccharides was added enough 95% ethanol to bring the final concentration of ethanol to 80% which resulted in the precipitation of these pectic polysaccharides. The precipitation was allowed to continue for two days at room temperature. The precipitated polysaccharides were recovered by centrifugation at 15,000 rpm for 1 h, dissolved in water and lyophilized. From a grapefruit weighing approximately 121 g, 2.5 g of pectic polysaccharides were recovered. Chemical analysis of the pectic polysaccharides thus obtained revealed the presence of only D-galacturonic acid and which accounted for 76% by weight of the total polysaccharides. The remaining 24% was accounted for by neutral sugar components: galactose, arabinose and trace amounts of rhamnose, xylose, mannose and glucose. This chemical composition is similar to the pectic polysaccharides isolated from lemon peel (23).

Labelling of Pectic Polysaccharides. We have successfully achieved the labelling of pectic polysaccharides by using two methods described below:

(a) Biosynthetic Labelling of Pectic Polysaccharides. Myo-inositol has been shown to be the precursor of oronosyl and pentosyl units of cell wall polysaccharides found in grapefruit (42). Labelled myo-inositol-2-³H (Amersham Searle, Inc.) was supplied to ripening grapefruit (the size of a golf ball) by placing the cut surface of fruit stem in a small vial containing the label. After the labelled myo-inositol had been taken up, distilled water was added to keep the cut fruit stem submerged, and the grapefruit was allowed to metabolize the label for a period of four days. The labelled grapefruit was then successively extracted with hot 80% ethanol, chloroform:methanol (1:1v/v) and finally with 0.2 M EDTA to solubilize pectic polysaccharides. These extraction procedures are described above in detail. Chemical analysis of the pectic polysaccharides following hydrolysis by colorimetric and gas chromatographic techniques, demonstrated the presence of D-galacturonic acid as the major component, constituting approximately 76% of the total sugars found in the pectin. The remaining 24% was consisted of rhamnose, arabinose, xylose, mannose, galactose, glucose at 16, 33, 13, 3, 31 and 2%, respectively, of the total neutral sugar components. It should be pointed out at this point that the chemical nature of these pectic polysaccharides was found to be identical with the pectic polysaccharides obtained from mature grapefruit albedo as described above.

The specific radioactivity of the labelled pectic polysaccharides thus obtained was about 8000 cpm/mg of polysaccharides, counting efficiency of ³H being 30%. Of the total radioactivity incorporated, almost 50% was localized in the galacturonic acid

residues whereas the remainder was accounted for by arabinose (21%), galactose (21%) and xylose (8%) residues. Results from these studies clearly demonstrated that labelled myo-inositol may successfully be used to incorporate the label in pectic polysaccharides found in grapefruit.

(b) Labelling of Pectic Polysaccharides Using Galactose Oxidase Tritiated Potassium Borohydride. Although we did achieve the labelling of citrus pectic polysaccharides by supplying labelled myo-inositol to ripening grapefruit as described above, the technique was expensive and time-consuming. Therefore, an alternate method for labelling of pectic polysaccharides was developed (43). The merit of this method lies in its rapidity, specificity and yield of labelled pectic polysaccharides with high specific radioactivity.

It has been established by several investigators and summarized in the background section of this proposal that, although pectic polysaccharides are primarily composed of polymers of galacturonic acid, neutral sugars such as galactose, arabinose and rhamnose also make up a considerable portion of these heterogeneous polysaccharides. The nature of glycosidic linkage appears to be 1-4 for galacturonans and galactans and 1-5 for arabans, suggesting therefore that, like the carboxyl group of galacturonic acid, the primary alcohol group of galactose is free and not involved in the formation of glycosidic linkages between various sugar residues found in pectic polysaccharides. This knowledge was utilized in the experiments described here.

Successful labelling of primary alcohol groups of galactose residues was achieved by first treating commercially available grapefruit pectin (Lykes-Pasco, Dade City FL) reprecipitated in 80% of ethanol in our laboratory before use, or grapefruit pectin prepared in our laboratory as described above, with galactose oxidase (Sigma Chemical Co.) in order to enzymatically modify the primary alcohol groups of galactose residues to an aldehyde group followed by the reduction of aldehyde group thus formed back to primary alcohol group with the concomitant introduction of ^3H by using tritiated potassium borohydride (Amersham Searle, Inc.) as a reducing agent. Examination of labelled pectic polysaccharides demonstrated the presence of label in the galactose residues. Although the successful labelling of primary alcohol group of galactose and/or galactosaminoglycans of animal and/or bacterial origin has been achieved by several investigators by this method originally described by Morell et al (44), to our knowledge, the present technique is the first to describe the use of Morell's method in labelling pectic polysaccharides.

Fractionation of Pectic Polysaccharides. A number of complex polysaccharides differing in their physical and chemical characteristics are found in pectin. This heterogeneous nature of pectin has been reviewed earlier. In order to characterize these

various polysaccharides, a combination of a number of fractionation procedures such as anion-exchange chromatography, molecular sieve chromatography and moving-boundary electrophoresis were employed to achieve the fractionation of pectic polysaccharides.

(a) Fractionation of Labelled Pectic Polysaccharides. In our laboratory, we have recently attempted the fractionation of labelled pectic polysaccharides. These labelled polysaccharides were obtained following administration of labelled myo-inositol to ripening grapefruit through the cut fruit stem, as described earlier. DE-52 diethyl aminoethyl cellulose ion exchanger was poured in a column (1.5x24 cm) and equilibrated with 0.025 M sodium phosphate buffer, pH 6.0. Labelled pectic polysaccharides (50 mg) having approximately 400,000 cpm were dissolved in 30 ml of equilibrating buffer and then loaded onto the column. The column was washed with 200 ml of equilibrating buffer and then eluted with a linear gradient ranging from 0.025 M to 0.5 M sodium phosphate, pH 6.0, buffer, 500 ml each. At the end of the gradient run, the column was finally washed with 200 ml of 1 M sodium phosphate buffer, pH 6.0. Fractions were collected and an aliquot from each fraction was assayed for radioactivity. The examination of results obtained from these studies suggests the presence of label in at least eight partially or completely resolved pectic polysaccharides. More than 95% of the total radioactivity loaded onto the column was recovered in these eluted pectic polysaccharides having different ion-exchange properties (42),

(b) Fractionation of Commercially Available Pectins. In another series of experiments we have attempted fractionation of commercially available citrus pectin by employing a stepwise elution method. To achieve this, grapefruit pectin was subjected to chromatography using DE-52 cellulose. Elution of the column with increasing salt concentrations resulted in the resolution of four chemically distinct pectic polysaccharides. These polysaccharides, eluted sequentially with 0.025, 0.1, 0.25 and 0.5 M sodium phosphate buffer, pH 6.0, composed 13, 5, 62 and 20%, respectively, of the total pectin subjected to chromatography. No striking differences in the galacturonic acid content, which ranged from 70-80% of these polysaccharides, were observed. However, polysaccharides eluted sequentially with increasing salt concentrations showed a noticeable decrease in the degree of methylation of their galacturonic acid residues. In addition, the neutral sugar composition of these individual pectic polysaccharides was different. Of interest was the neutral sugar composition of polysaccharides eluted with the highest salt concentration. This highly acidic polysaccharide was also high in rhamnose content. The results obtained from these studies affirm the heterogeneous nature of pectin, and suggest the presence of a unique, rhamnose-rich polysaccharide in grapefruit pectin.

Biochemical Basis of Observed Interaction Between Pectin and Lipoprotein. These studies await further research. A prerequisite to these studies is the establishment of the chemistry and structure of various polysaccharides found in citrus pectin. As described above, these studies are in progress and will eventually lead to the isolation of several pure citrus pectic polysaccharides of defined chemistry.

In our study, formation of insoluble complexes between pectin, a heterogeneous mixture of a number of neutral and acidic polysaccharides, and lipoprotein was studied. The basic limitation with the formation of insoluble complexes is that it is difficult to quantitate the said interaction. Furthermore, the observed interaction between pectic polysaccharides and lipoprotein is at a pH which is not physiological. We, therefore, are attempting to study this interaction under physiological conditions and by use of buffer systems which are devoid of cations, in order to facilitate formation of soluble complexes. In addition, by using labelled pectic polysaccharides, studies resulting in the elucidation of kinetics, specificity and nature of the interaction between labelled pectic polysaccharides and lipoprotein will be performed.

The specificity of the interaction will be determined with regard to a single labelled pectic polysaccharide by studying the competitive ability of other unlabelled pectic polysaccharides of known characteristics on the formation of labelled complexes. The data obtained from these studies will also be evaluated to determine the influence of change in molecular weight and/or structure of individual pectic polysaccharides on the binding to low density lipoprotein. Similar experiments will also be carried out to study the competitive inhibition of well established interactions between low density lipoprotein and various glycosaminoglycans caused by pectic polysaccharides. The observation of inhibition of interaction between lipoprotein and glycosaminoglycans by pectic polysaccharides may suggest, at least in vitro, the possibility of a basis by which dissolution of atherosclerotic plaques could be achieved by pectic polysaccharides.

Our preliminary experiments suggest that, like the interaction between glycosaminoglycans and lipoproteins, the interaction between pectin and lipoprotein is also caused by the electrostatic attraction between polyanionic pectin and cationic lipoprotein. To elucidate this hypothesis, the charge profile of lipoproteins will be altered chemically and the influence of these alterations on the interaction with pectic polysaccharides at optimum experimental conditions will be determined.

The significance of the observed interaction between pectin and LDL, and further biochemical elucidation of this interaction, has direct relevance to the etiology and/or cure of atherosclerosis. The role of dietary pectin in lowering of serum and liver cholesterol levels is well established. However, the biochemical basis by which dietary pectin, which is composed of

a number of complex polysaccharides, causes this lowering of cholesterol levels remains elusive. These studies will provide an answer to this problem. In addition, these studies may result in the identification of a unique and biologically active polysaccharide found in pectin, which may be solely responsible for lowering of cholesterol levels. Furthermore, the results obtained from these studies may provide us with clues to achieve dissolution of atherosclerotic plaques, since LDL is the major ingredient of these plaques and pectic polysaccharides interact with the LDL.

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Medical and Nutritional Aspects of Citrus Bioflavonoids

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In 1936 the discovery that the flavanone fraction of lemons exerted therapeutic effects on abnormal capillary permeability and fragility ushered in the era of research on role of flavonoids in the mammalian body (1). Flavonoids were proposed to be vitamins (Vitamin P) necessary to maintain the capillaries, but the vitamin concept was never substantiated and in 1950 Vickery et al. (2) recommended the term Vitamin P be discontinued (term Vitamin P was replaced by bioflavonoid). Subsequent research had failed to confirm early indications that abnormal capillary permeability and fragility were due to a bioflavonoid deficiency but showed these capillary defects accompanied many diseases and stresses (3,4). In 1954 Martin (4) stated "There is no disease state in which the capillaries are not detrimentally modified and conversely there are no disease states that will not benefit by assuring proper capillary strength and integrity." In 1955 Hendrickson and Kesterson (5) listed more than 50 diseases in which bioflavonoids reportedly showed beneficial effects on capillaries or the disease process itself. However, slight and inconsistent effects along with failure to establish a mode of action led to controversies concerning the therapeutic effectiveness of bioflavonoids (6). In 1963 Freedman and Merritt (7) aptly summarized the literature with the statement: "Following Szent-Gyorgyi's original observations on the effect of his Vitamin C or citrin preparations, investigations by the many workers produced an experimental and clinical literature of contradictory thought and observation." However, in recent years, evidence has accumulated on the presence of highly active methoxylated flavonoids and steroids in citrus extracts, and the Vitamin P flavonoids have been found to exhibit a trimodal action. These findings have shed considerable light on the contradictory literature.

Variations in Citrus Extracts

The original work of Szent-Gyorgyi and co-workers was done with a crude lemon extract containing primarily hesperidin and

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eriodictyol, and the above workers had difficulties, using crude extracts, reproducing their initial observations (8). In 1943 work still centered on trying to identify the active component in citrus extracts. For example, Higby (9) claimed crude extracts containing hesperidin to be active, while purified hesperidin was inactive, but in 1945 Scarborough (10) reported that hesperidin exhibited biological activity. In 1963 Freedman and Merritt (7) fractionated a citrus flavonoid complex and identified in addition to hesperidin and naringin, a hexamethoxylated flavone (nobiletin), a pentamethoxy flavone (not tangeretin, probably sinensetin) and an unidentified compound with reducing properties. In contrast to hesperidin and naringin, which exhibited no antiinflammatory activity, the methoxylated flavones showed strong antiinflammatory activity displaying a broader inhibitory potential than either cortisone or ACTH. Later, Biondi (11) and Biondi et al. (12) reported the isolation from mixed citrus bioflavonoids of a steroid fraction which in minimal doses showed cortisone-like activity in the rat and guinea pig. Freedman and Merritt (7) pointed out that the amount of their highly active substances varied according to the methods of extraction and preparation employed. Importantly, the above work showed the occurrence in citrus of small amounts of highly active substances distinct from the abundant vitamin P flavonoids, and the amount of these active substances recovered with the P compounds depended on the methods employed. Undoubtedly, the variability in bioflavonoid preparations from citrus extracts has contributed to the confusion in the literature.

Trimodal Action of Bioflavonoids

In addition to variations in citrus extracts, there are features of bioflavonoid effects on the mammalian body which may have contributed greatly to the controversy concerning flavonoid action. In 1940 Yosida (13) and in 1955 Rinehart (14) reported effects of hesperidin on rheumatic fever. Yosida determined the erythrocyte sedimentation rate (ESR) before and after injecting hesperidin into patients. In 26 sets of determinations the ESR was retarded in 19, unchanged in 3 and accelerated in 4 cases. Rinehart administered hesperidin to 26 patients, 22 showed a decreased ESR, 1 an unchanged and 3 an increased ESR. Hence, by the usual interpretation that an increased ESR indicates pathology, hesperidin in some cases showed beneficial effects, in others no effect and in still others made the disease worse. These are indeed confusing results. However, later research provided data for a different interpretation of the above results, i.e., hesperidin did not act on the disease process but altered the ESR by direct action on blood cells (15,16,17,18). In 1966 Robbins (15) reported that hesperidin, naringin and rutin reduced the intravascular erythrocyte adhesion accompanying Vitamin C deficiency in the guinea pig. The above findings

indicate an effect on the ESR since increased erythrocyte aggregation is the major factor underlying an increased ESR. This was borne out in subsequent work in which bioflavonoids added to blood in vitro reduced the ESR by disaggregation of blood cells (16,17,18). Other workers reported action of flavonoids on blood cells. In 1971 Srinivasan et al. (19) reported that flavonoids reduced blood cell aggregation. In 1973 Ten Cate et al. (20) and in 1975 Van Haeringen (21) reported that the semi-synthetic derivatives of rutin O(β -hydroxyethyl) rutosides reduced red cell and platelet aggregation. It is of considerable interest that the naturally occurring methoxylated flavones of citrus were several fold more active against blood cell aggregation than hydroxylated flavonoids (16,17), and the substitution of ethoxy groups on rutin greatly increased its activity against blood cell adhesion (17,21).

When several bioflavonoids were compared for blood cell disaggregating activity against the high ESR of normal horse blood (rouleaux formatin), hesperidin, rutin and quercetin showed slight but consistent retarding effects on the ESR while the highly methoxylated flavones, sinensetin, nobiletin, heptamethoxyflavone showed several-fold greater retarding activity than the above compounds, but naringin consistently accelerated the ESR of horse blood (16,17,18). However, when the above bioflavonoids were tested in vitro against the increased ESR occurring in a wide variety of human diseases, the hydroxylated and less active methoxylated bioflavonoids exhibited a trimodal effect (22), i.e., inhibited the ESR in some cases, showed no effect in others and accelerated the ESR in still others; the same effect Yosida and Rinehart (13,14) observed when hesperidin was tested in vivo. Hesperidin which had consistently inhibited, and naringin which had consistently accelerated the ESR of horse blood, both showed a trimodal effect on the ESR of blood taken from a population of humans afflicted with a wide variety of diseases (22). It should be emphasized that against one kind of erythrocyte adhesion (rouleaux formation of horse blood) the Vitamin P flavonoids were consistent in action but inconsistent in action against erythrocyte adhesion occurring in a wide variety of human diseases. Thus, the antiadhesive action of bioflavonoids on erythrocytes depends on the kind of bonding between cells, which according to Easty and Mercer (23), depends on the agglutinating agent. However, the hexamethoxylated flavonoid nobiletin in several studies on erythrocyte adhesion in a wide variety of human diseases has not been found to significantly accelerate the ESR, while sinensetin showed significant accelerating action in one study (22).

The trimodal action of bioflavonoids on the ESR is not without precedent. Dintenfass (24) found that a pyrimidine type drug of the Persantin family showed a trimodal action on the ESR and, the accelerating effect was related to ABO blood type. Blood containing the A antigen showed a statistically significant increase in accelerated ESR. In contrast, bioflavonoids exhibited a significantly higher rate of accelerated ESR's in blood contain-

ing the B antigen (25). Whether the bioflavonoids and pyrimidine drug are influenced in their response via the mucopolysaccharides on the cell membrane which determine blood type or are influenced via alterations of plasma constituents which may respond to blood type is not known. Both concentrations of some plasma constituents and response of blood cells to plasma constituents are influenced by ABO blood type (26,27). Whether blood type antigens influence adhesive bonds between erythrocytes under various conditions of disease and stress remains to be determined.

While the trimodal action of bioflavonoids on blood cells sheds considerable light on the nature of their action in the body, questions arise as to what extent action on blood cells and blood rheology explains, in particular, their action on abnormal capillary permeability and fragility and therapeutic action in a wide variety of diseases. It should be noted that while a great number of effects of bioflavonoids have been reported, the mechanism of action proposed are relatively few, i.e., protect ascorbic acid against oxidation (6,28), prevent epinephrine oxidation (29), stimulate the pituitary adrenal axis (30), inhibit a variety of enzymes such as aldose reductase (31) or membrane bound ATP ases (32), selectively inhibit phosphodiesterase (33), act in cross-linking of elastin (34), stimulate the reticuloendothelial system (35), act as vasotropic drugs (36), inhibit blood cell aggregation (15-21) and prevent lactacidotic rigidification of the red cell (37); the latter two effects indicate action on blood rheology. There is little evidence to support any of the above mechanisms, except the action of bioflavonoids on blood rheology, as explaining their effects on abnormal capillary permeability and fragility over a broad range of conditions and beneficial effects in a wide variety of diseases.

Blood Rheology and Capillary Fragility

As noted earlier, bioflavonoids first attracted attention as agents that acted on abnormal capillary permeability and fragility which was a characteristic finding in scurvy (6). However, subsequent research revealed the above capillary defects were not restricted to scurvy. Brown (3) reported the occurrence of a decreased capillary resistance in a wide variety of diseases. He found that infections were often present at the time when low capillary resistance was observed. Consistent with the above capillary defects in disease, an increased blood cell aggregation and ESR is also a generalized response of the body to infection and disease (38). Erythrocyte aggregates interfere with blood flow through the small vessels and contribute to increased blood viscosity in larger vessels. Both phenomena cause adverse physiological effects. For example, the blood high viscosity syndrome includes fatigability, bleeding from mucous membranes, retinal vein enlargement and hemorrhage, neurological abnormalities and heart failure (39). (Several parameters can contribute to the

above condition, in addition to erythrocyte aggregation, erythrocyte concentration, rigidity of the erythrocytes and plasma viscosity are involved in blood viscosity (40)). The resistance of blood to flow reduces capillary perfusion (41) with decreased rates of oxygen delivered to the capillary endothelium. According to Knisely et al. (42) anoxic endothelium increases in permeability. This is consistent with the observations of Landis (43) that decreased blood flow causes a rapid increase in capillary permeability, but on resumption of flow, permeability rapidly returns to normal. Under two kinds of experimental conditions, Robbins (15, 44) found an inverse relation between blood cell aggregation and capillary resistance, i.e., as blood cell aggregation increased capillary resistance decreased and when aggregation decreased capillary resistance increased. It should be noted here that bioflavonoids have been proposed to be vasotropic drugs (36), that is, prevent loss of endothelial cells from blood vessel walls. O'Neill (45) reported that a reduced blood flow caused a peeling of the venous endothelium. Thus, impaired blood flow appears capable of causing abnormal capillary permeability and fragility. Further, the trimodal action of bioflavonoids on blood cells offers an explanation for inconsistent action on the capillaries. However, it cannot be overlooked that bioflavonoids may act directly on the capillary wall, and the same factors that affect reaction of flavonoids with blood cells influences reaction with the capillary membranes. It should be noted that bioflavonoids bind reversibly to both erythrocytes (20) and plasma proteins (46). The latter action stimulates phagocytosis in certain edemas (47) thereby exerting a therapeutic effect. Also, note that in the above type edemas the action of bioflavonoids is not on changes in permeability of the capillary wall.

Therapeutic Effects in Disease

The action of bioflavonoids on blood rheology appears to explain their reduction of symptoms in a wide variety of diseases. Increased blood cell aggregation and ESR is a generalized response of the organism to disease and trauma (38,42) and reportedly causes adverse effects such as interference with gaseous exchange in tissues, accumulation of acid metabolites, slowed healing of wounds, degenerative changes in parenchymatous organs and promotion of thrombosis (48). Fajers and Gelin (49) reported that kidney, liver and heart damage resulted from severe blood cell aggregation. In agreement, Bergentz et al. (50) stated that if erythrocyte aggregation was severe and of long duration it caused organ enlargement and infarction. According to Fahraeus (51) eclampsia is a disease of checked microcirculation by red cell aggregates. Further, erythrocyte aggregation may interact with other disease processes to increase symptoms (52,53). Thus, action of bioflavonoids on blood cell aggregation could explain their beneficial effects in a wide variety of diseases.

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Perhaps at this point a question should be raised as to whether acceleration of the ESR by flavonoids is an adverse effect. In an investigation of the accelerative action of bioflavonoids, the ESR increased 6 to 23% depending on the flavonoid (54). Based on an average ESR of 26.8 mm per hr. in the group of hospitalized patients under study, the maximum average accelerated ESR would reach 33.0 mm per hr. In contrast, the ESR may reach 50 mm or more per hr. in certain diseases or traumas. Apparently, acceleration of the ESR is caused by flavonoid bridging between red cells and may selectively involve aged red cells similar to the action of polylysine (55). The bridged cells may then be removed from the circulation by the phagocytic cells of liver spleen and bone marrow (56). The phagocytic action may continue under some conditions to the point of anemia (56), but, with flavonoids the accelerative action is associated with high red cell concentrations and decreases when the concentration falls to about 40 volumes percent; area of maximum effective tissue perfusion (57). From this, it was postulated that bioflavonoids might exert a regulatory action on the hematocrit. However, the above action is not proven, but the phenomenon deserves further attention since an elevated hematocrit reportedly is associated with ischemic heart disease (58).

Intact Organism, Physiological, Biochemical Levels

When bioflavonoids are tested against biochemical parameters such as effect on isolated enzymes, they show consistent action. For example, quercetin inhibits membrane-bound ATPases, and there are several reports, in agreement, that quercetin in vitro inhibits ATPases from different species (32). Also, there does not appear to be controversy concerning inhibitory action of quercetin on other enzymes such as aldose reductase or catechol-O-methyltransferase (31,59). When we move to the physiological level and determine effect of bioflavonoids on blood cell adhesion, there is consistent activity when the compounds are tested against a particular uniform type of adhesion such as rouleaux of horse blood, but they are inconsistent in action when tested against different kinds of bonds between aggregated cells as may occur in human diseases (22,23,42,48). The controversies in the literature have arisen at the level of the intact organism when bioflavonoids were tested against a wide variety of diseases, vascular abnormalities, anaphylactic shock or non-specific stresses. For example, as noted earlier when hesperidin was injected into patients, three kinds of effects were noted, and quercetin described above as consistent in action at the biochemical level exerted a trimodal action on erythrocyte aggregation (22). Thus, there are factors operating in the intact organism which modify bioflavonoid action.

Specificities in Bioflavonoid Action

When a variety of bioflavonoids are tested against a biological parameter, usually several flavonoids will show some activity exhibiting their effects as a family of curves differing significantly in activity. For example, over 40 flavonoids showed some inhibitory activity on aldose reductase, but quercetin and quercitrin were most active (31). Nineteen flavonoids were observed to inhibit catechol-0-methyltransferase of human liver, but those with 0-dihydroxy structure at the side ring showed the greatest effect (59). The 0 (β -hydroxyethyl) rutosides (HR) are semisynthetic derivatives of naturally occurring rutin. The crude preparation consists of the following: 7-10% of 5,7,3',4'-tetra HR; 60-65% of 7,3',4'-tri HR; 5-8% of 5,7,4'-tri HR; 8-12% of 7,4 di HR and 5-8% of 4' mono HR. A mixture of mono/di HR has the greatest inhibitory effect on platelet aggregation (60), but the tetra derivative has the greatest effect on erythrocyte aggregation (21). The methoxylated bioflavonoids of citrus showed the following order of activity (from high to low) against the high ESR of horse blood, i.e., sinensetin (pentamethoxy), nobiletin (hexa), heptamethoxyflavone (hepta), tangeretin (penta), tetra-0-methylscutellarein (tetra), tri-0-methylapigenin (tri) and hesperidin (mono). Sinensetin has methoxyl groups at 5,6,7,3',4' whereas nobiletin (5,6,7,8,3',4' hexamethoxyflavone) differs by a methoxyl at the 8 position and is significantly ($P < 0.01$) less active. A methoxyl group at the 3 position, in addition to the 8th, reduced activity still further as shown by 3,5,6,7,8,3',4' heptamethoxyflavone. Tangeretin 5,6,7,8,4' pentamethoxyflavone lacks a methoxyl group at the 3' position which represents the structural difference between nobiletin and tangeretin, and nobiletin is significantly ($P < 0.01$) more active. Tangeretin differs from sinensetin by shifting a methoxyl group from the 3' position of sinensetin to the 8 position of tangeretin with a several-fold drop in activity (22). However, when sinensetin, nobiletin and heptamethoxyflavone were tested in human blood taken from patients with a variety of diseases (61), the three compounds showed equal activity in 44.8% of the cases and heptamethoxyflavone showed significantly greater activity than the other two flavones in 2.4% of the cases. Apparently, certain structural configurations are more effective against certain kinds of adhesive bonds between erythrocytes.

Citrus Bioflavonoids and Disease

The highly methoxylated flavones found in food plants are almost unique to citrus, and they are biologically an unusually active group of compounds. Several methoxylated flavones have been isolated from citrus species (62,63,64), Table 1, with most attention given to biological activity of nobiletin, tangeretin, sinensetin, heptamethoxyflavone and tetra-0-methylscutellarein. Generally, methoxylated flavonoids exhibit much higher biological activity than their methoxyl free counterparts. Also they are

Table I
Flavonoids Isolated from Citrus Species

Flavones

Apigenin 7- β -rutinoside (5,7,4'-trihydroxyflavone 7- β -rutinoside)
 Auranetin (3,6,7,8,4'-pentamethoxyflavone)
 Chrysoeriol glycoside (5,7,4'-trihydroxy-3'-methoxyflavone glycoside)
 Diosmin (5,7,3'-trihydroxy-4'-methoxyflavone 7- β -rutinoside)
 Fortunellin (5,7-dihydroxy-4'-methoxyflavone 7- β -neohesperidoside)
 Hexo-0-methylgossypetin (3,5,7,8,3',4'-hexamethoxyflavone)
 Hexo-0-methylquercetagenin (3,5,6,7,3',4'-hexamethoxyflavone)
 Isolimocitrol 3- β -D-glucoside (3,5,7,3'-tetrahydroxy-6,8,4'-trimethoxyflavone 3- β -D-glucoside)
 Isorhamnetin glycoside (3,5,7,4'-tetrahydroxy-3'-methoxyflavone glycoside)
 Isosinensetin (5,7,8,3',4'-pentamethoxyflavone)
 Kaempferol glycoside (3,5,7,4'-tetrahydroxyflavone glycoside)
 Limocitrin 3- β -D-glucoside (3,5,7,4'-tetrahydroxy-8,3'-dimethoxyflavone 3- β -D-glucoside)
 Limocitrol 3- β -D-glucoside (3,5,7,4'-tetrahydroxy-6,8,3'-trimethoxyflavone 3- β -D-glucoside)
 Luteolin 7- β -neohesperidoside (5,7,3',4'-tetrahydroxyflavone)
 Natsudaidin (3-hydroxy-5,6,7,8,3',4'-hexamethoxyflavone)
 Neodiosmin (5,7,3'-trihydroxy-4'-methoxyflavone 7- β -neohesperidoside)
 Nobiletin (5,6,7,8,3',4' hexamethoxyflavone)
 Rhoifolin (5,7,4'-trihydroxyflavone 7- β -neohesperidoside)
 Rutin (3,5,7,3',4'-pentahydroxyflavone 3- β -rutinoside)
 Sinensetin (5,6,7,3',4'-pentamethoxyflavone)
 Sudachitin (5,7,4'-trihydroxy-6,8,3'-trimethoxyflavone)
 Tangeretin (5,6,7,8,4'-pentamethoxyflavone)
 Tetra-0-methylisoscuteallarein (5,7,8,4'-tetramethoxyflavone)
 Tetra-0-methylscuteallarein (5,6,7,4'-tetramethoxyflavone)
 Xanthomicrol (5,4'-dihydroxy-6,7,8-trimethoxyflavone)
 3'-Demethoxysudachitin (5,7,4'-trihydroxy-6,8-dimethoxyflavone)
 3-Hydroxy-5,6,7,3',4'-pentamethoxyflavone
 3,5,6,7,8,3',4'-heptamethoxyflavone
 5-Hydroxy-3,6,7,8,3',4'-hexamethoxyflavone
 5-Hydroxy-3,7,8,3',4'-pentamethoxyflavone
 5-Hydroxy-7,8,3',4'-tetramethoxyflavone
 5-0-Desmethyltangeritin (5-hydroxy-6,7,8,4'-tetramethoxyflavone)
 5-0-Desmethoxynobiletin (5-hydroxy-6,7,8,3',4'-pentamethoxyflavone)
 5,4'-Dihydroxy-6,7,8,3'-tetramethoxyflavone
 5 Hydroxyauranetin (5-hydroxy-3,6,7,8,4'-pentamethoxyflavone)

Flavanones

Citromitin (5,6,7,8,3',4'-hexamethoxyflavanone)
 Dihydrokaempferol glycoside (3,5,7,4'-tetrahydroxyflavanone glycoside)

- Eriodictyol new name Eriocitrin (5,7,3',4'-tetrahydroxyflavanone 7- β -rutinoside)
Hesperidin (5,7,3'-trihydroxy-4'-methoxyflavanone 7- β -rutinoside)
Isosakuranetin 7- β -rutinoside (5,7-dihydroxy-4'-methoxyflavanone 7- β -rutinoside)
Naringin (5,7,4'-trihydroxyflavanone 7- β -neohesperidoside)
Naringin 4'- β -D-glucoside (5,7,4'-trihydroxyflavanone 7- β -neohesperidoside 4'- β -D-glucoside)
Naringenin 7- β -rutinoside (5,7,4'-trihydroxyflavanone 7- β -rutinoside)
Naringenin 7- β -rutinoside 4'- β -D-glucoside (5,7,4'-trihydroxyflavanone 7- β -rutinoside 4'- β -D-glucoside)
Neohesperidin (5,7,3',4'-tetrahydroxyflavanone 7- β -neohesperidoside)
Neohesperidin (5,7,3'-trihydroxy-4'-methoxyflavanone 7- β -neohesperidoside)
Poncirin (5,7-dihydroxy-4'-methoxyflavanone 7- β -neohesperidoside)
5-O-Desmethylcitromitin (5-hydroxy-6,7,8,3',4'-pentamethoxyflavanone)

C-Glycosylflavones

- Isomargariten (5,7-dihydroxy-4'-methoxy-6C- β -neohesperidosylflavone)
Margariten (5,7-dihydroxy-4'-methoxy-8-C- β -neohesperidosylflavone)
2''-O- β -D-xylosylvitexin (5,7,4'-trihydroxy-8 (2''-O- β -D-xylosyl-C- β -D-glucosyl) flavone)
6-C- β -D-Glucosyldiosmetin (5,7,3'-trihydroxy-4'-methoxy-6-C- β -D-glucosylflavone)
6,8-Di-C- β -D-glucosylapigenin (5,7,4'-trihydroxy-6,8-di-C- β -D-glucosylflavone; Vicenin-2)
6,8-Di-C-Glycosyldiosmetin (5,7,3'-trihydroxy-4'-methoxy-6,8-di-C-glycosylflavone)
8-C- β -D-Glucosyldiosmetin (5,7,3'-trihydroxy-4'-methoxy-8-C- β -D-glucosylflavone)

Anthocyanins

- Cyanidin 3- β -D-glucoside (3,5,7,3',4'-pentahydroxyflavylium 3- β -D-glucoside)
Cyanidin 3,5,-di- β -D-glucoside (3,5,7,3',4'-pentahydroxyflavylium 3,5-di- β -D-glucoside)
Delphinidin 3- β -D-glucoside (3,5,7,3',4',5'-hexahydroxyflavylium 3- β -D-glucoside)
Peonidin 5- β -D-glucoside (3,5,7,4'-tetrahydroxy-3'-methoxyflavylium 5- β -D-glucoside)
Petunidin 3- β -D-glucoside (3,5,7,3',4'-pentahydroxy-5'-methoxyflavylium 3- β -D-glucoside)
Ref. Table taken from reference no. 62; see references 63 and 64 for further information.

more resistant to bacterial degradation in the intestinal tract (65,66,67). Reportedly, the methylation of hydroxylated flavonoids can be carried out in the intact animal by the liver enzyme catechol-O-methyltransferase which catalyzes introduction of methyl groups into B ring hydroxyls in flavonols (68,69). Reportedly, quercetin given orally to rats was methylated in the 3' position (68). This conversion increases the activity of the compound and raises questions as to whether certain methoxylated compounds have an indispensable role in the body.

Certain bioflavonoids may play a preventive role against cardiovascular diseases. Some citrus and other bioflavonoids have been demonstrated to reduce serum cholesterol levels and to affect fatty acid metabolism (70,71,72). The strong antiadhesive action on red cells and platelets of highly methoxylated flavones such as nobiletin, which also demonstrates antithrombogenic activity (73), indicates an important role in blood rheology and tissue perfusion. The antiadhesive action may indicate a preventive role in atherosclerosis since there is evidence that reduced perfusion of the vascular wall may interact with serum lipids to promote atherogenesis (74).

Also, a role is indicated for citrus bioflavonoids against certain carcinogenic and cytotoxic agents. The methoxylated flavones, tangeretin and nobiletin, reportedly induce aryl hydrocarbon hydroxylase activity, enzymes which detoxify polycyclic carcinogens (75,76). Most hydroxylated bioflavonoids have little, if any, inducing activity on aryl hydrocarbon hydroxylase. The inducing capacity of methoxylated flavones in most instances parallels their anti-tumor activity which is demonstrated both *in vivo* and *in vitro* against several strains of human carcinoma cells (77). Recently, Swartz and Rate (78) found that four bioflavonoids (quercetin, fisetin, nobiletin, tangeretin) protected cultured rat liver cells against aflatoxin B₁-induced cytotoxicity and inhibited binding of aflatoxin B₁ to cellular DNA. The two methoxylated compounds showed greater protection than the hydroxylated flavonoids. The hydroxylated bioflavonoid, quercetin, reportedly inhibits the high aerobic glycolysis in tumor cells *in vitro* (but this effect is negated by serum albumin and bicarbonate (79,80)). The effect is ascribed to inhibition of membrane bound ATPases that generate the ADP and inorganic phosphate required for glycolysis. Also, quercetin affects cyclic AMP by inhibition of phosphodiesterase which is involved in control of cell growth. In several types of malignant cells a low level of cyclic AMP was observed and believed to be responsible for loss of contact inhibition and uncontrolled growth of tumor cells. The decreased concentration of cyclic AMP in malignant cells may be due to reduced activity of adenylate cyclase, (the enzyme responsible for formation of cyclic AMP) or an increased activity of phosphodiesterase, (the enzyme which catalyzes its degradation) (81). It should be noted that while quercetin acts consistently in the above systems, it shows a trimodal action on blood cells in human diseases (22).

In addition to the kinds of activity enumerated above, the highly methoxylated bioflavonoids exhibit a rather strong anti-bacterial, antifungal, antiviral activity (82-86). The compounds have shown inhibitory activity against organisms such as E. coli, Typhi, Salmonella Typhi, Shigella Dysenteriae, Staphylococcus, B. Abortus Bang, influenza Virus A and rhinoviruses. Some of the compounds were active in concentrations as low as 2.5 μ g per liter.

Are Certain Flavonoids Essential in the Diet?

The inevitable question arises: Are bioflavonoids essential in the diet? Kuhnau (63) in a review called flavonoids semi-essential nutrients and stated that flavonoids have shown in the mammalian organism a great number of specific effects valuable or even decisive for maintaining health and well-being. Certain flavonoids have been reported to be feeding stimulants for certain insects (87,88,89), growth factors for the cricket (90), vitamins for the rat with a deficiency in the diet causing microscopic structural alterations in tissues (91) and a decreased metabolism of drugs (92). Nevertheless, it has not yet been demonstrated that in the absence of certain bioflavonoids in the diet an animal sickens and dies; a rigorous and necessary definition of essentiality. However, from another point of view, the methoxylated flavones with their broad spectrum of activity might be interpreted as a defense system against disease transferred from the plant to the animal through the diet with an action directed against biochemical and physiological mechanisms such as red cell adhesion and impaired microcirculation that may be decisive in prevention of degenerative processes (93). Thus the question of essentiality in the case of man may hinge on the question of whether in the absence of these compounds in the diet physiologic aberrations arise which uncorrected are eventually manifested as degenerative diseases. Hence, if humans consuming certain bioflavonoids survive for a longer time than those without the bioflavonoids, then the compounds have met the rigorous test of essentiality; without the compounds man sickens and dies. However, in such a situation, cause-effect associations are difficult to make since long periods of time may elapse between initiation of the deficiency (or long-term ingestion of slightly active compounds) and the appearance of ill-health and death. Further, confusion may arise because similar deficiencies may be manifested in different people by different sets of symptoms. Thus, essentiality is difficult to demonstrate by the usual procedures.

Is a Theory Unifying Mode of Action Possible At Present?

The great number of flavonoids found in nature and the myriad of physiological effects appears to defy an unifying concept of mode of action. However, while flavonoids may exert a yet unde-

terminated nutrient role, present evidence suggests that flavonoids (some at least) act as a broad spectrum defense system with activity against disease in both plants and animals. We appear to be viewing in this case a primitive defense system generated in the plant with vestiges of activity still demonstrated in the animal body. There are evolutionary relations between plant and animal cells. Vitamins and certain amino acids synthesized only in plants are required by both plants and animals. Similarly, flavonoids synthesized only in plants may increase in concentration in response to invasion by pathogens or other adverse conditions (94, 95), thereby exerting a defensive role in the plant. Some basic similarities between metabolism in plant and animal cells underlie activity in the animal body. There is some support for the above point of view. In 1937 Schreiber and Elvehjem (96) reported that Vitamin P appeared to exert a vitamin-like action only in stress or emergency situations. Essentially in agreement, Hughes and Wilson (28) state that available evidence suggested that flavonoids are of greatest significance in restoring the status-quo to the animal body.

Possibly, the variety of flavonoid effects, i.e., trimodal action on blood cells or other parameters; potentiation or inhibition of enzymes; antibacterial, antifungal, antiviral activity; cytotoxic effects against tumor cells, etc. are manifestations of a fundamental physiochemical mode of action which involves a binding or reaction of flavonoids with specific sites on tissues, cells or cell constituents. Thus, flavonoid action occurs as an expression of molecular configuration, reactivity, nature and availability of specific binding or reactive sites in the body; a diverse system of protection. Specificity is indicated by the finding that the B antigen of the ABO blood groups is involved in the trimodal action on human blood cells (25).

Microorganisms which succumb to action of a specific flavonoid evidently have not had previous exposure to the flavonoid. However, animals have been exposed to a variety of flavonoids in their diet throughout their evolution and adaptations have occurred to where minimal binding or reactive sites are available in tissues of normal animals. However, the disruptive processes of diseases, traumas and stresses would appear to establish a basis for flavonoid action since these adverse conditions may cause alterations in tissue constituents such as morphological alterations in proteins with which flavonoids react to exert their reparative processes. For example, flavonoids bind (reversibly) to blood cells to exert their antiadhesive action (20), bind to abnormal proteins and stimulate phagocytosis to reduce edemas (47), bind to enzymes to alter activity (32), bind to microorganisms to exert inhibitory or lethal action (94). The exposure of binding sites in disease or stresses appears to be corroborated by findings that in rats on a flavonoid-free diet fine structural alterations occur in blood vessels and tissues (91). The anabolism involved in the reparative process, such as the above, may explain stimulation of the basal metabolic rate by flavonoids (65).

A specific disease or other damaging agent would not appear to result always in precisely the same alterations in tissue constituents thus, differences occur in reactive sites which suggests why a particular flavonoid does not always exhibit the same effect in a particular physiological or pathological condition. For example, Grigor'eva (97) in a study of effects of bioflavonoids on serum cholesterol observed that when the compounds were administered to 26 animals, serum cholesterol decreased in 18, did not change in 4 and increased in 4. The effects observed may be due to potentiation, no effect, or inhibition of enzymes which in turn depends on available reactive or binding sites on enzymes. Also, available binding sites and potentiation of enzymes may explain the growth promoting effects of flavonoids in certain insects.

A theory of flavonoid action as described above appears to explain several confusing features of their action, i.e., the wide variety of effects in the animal body, why a considerable number of flavonoids show activity, the trimodal action and the apparent inconsistent effects.

Summary

Subsequent to the discovery that the flavanone fraction of lemons exerted a therapeutic effect on abnormal capillary permeability and fragility, controversies arose concerning the identity of the active substance and the nature of its role in the body. The controversies have long hampered efforts to make effective use of bioflavonoids in nutrition and medicine, but research of recent years has shed considerable light on phenomena underlying inconsistent flavonoid action. Findings in the following areas have made substantial contributions to understanding bioflavonoid action:

1. In the early work on bioflavonoids, citrus extracts were used, and later research has revealed the presence of highly active methoxylated flavones in citrus. The amount of these flavones recovered along with the more abundant hesperidin, naringin, eriodictyol varies with the methods of extraction and purification used. Thus, bioflavonoid preparations by the many workers may have varied widely in activity.
2. Efforts to identify the most effective bioflavonoids have been frustrated by the multiplicity of flavonoids which have demonstrated some degree of activity against a particular biological parameter. The early criteria of bioflavonoid activity, i.e., action on capillary defects or beneficial effects in a wide variety of diseases lacked discriminatory sensitivity. Testing of bioflavonoids against biochemical parameters such as isolated enzymes or a simple uniform physiological parameter, such as rouleaux formation of normal blood, has shown that a variety of flavonoids will demonstrate their activity as a family of curves differing significantly in activity.

However, while many flavonoids may show activity, clinically useful compounds are few in number. For example, only nobiletin and sinensetin of citrus and the semi-synthetic tetra-ethoxy derivative of rutin are effective erythrocyte disaggregating agents; compounds of choice at present.

3. Bioflavonoids have been discovered to exert an antiadhesive action on blood cells. Erythrocyte adhesion is a general accompaniment of disease and trauma and has rheological implications. Hesperidin or other of the less active flavonoids administered to a series of patients or added to blood in vitro may show three kinds of activity, inhibit blood cell adhesion in some, no effect in others or accelerate adhesion in still others, i.e., a trimodal action. This characteristic feature of flavonoid action undoubtedly has been interpreted as an inconsistent effect. However, all flavonoids do not show a trimodal action. Also, there is considerable evidence linking rheological effects of bioflavonoids to their effects on capillary defects and beneficial effects in disease. Thus, the trimodal effects may explain the apparent inconsistent action against the above phenomena.
4. Research of recent years has moved bioflavonoids from a consideration of their effects on capillaries into a more central position in human health. Certain bioflavonoids intervene at the molecular level to potentiate or inhibit enzyme action or at the physiological level to affect rheology of blood. The broad spectrum of activity demonstrated by some of these methoxylated flavones appears to be directed towards processes which underlie degenerative diseases, at present our greatest killers.

This survey of present evidence indicates that we have arrived at a level of understanding of bioflavonoids to where we can begin to make effective use of these compounds. Bioflavonoids understood and applied in modern nutrition and medicine would appear to confer advantages in increased resistance to a variety of diseases against which we do not have effective measures at this time.

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Limonin and Limonoids

Chemistry, Biochemistry, and Juice Bitterness

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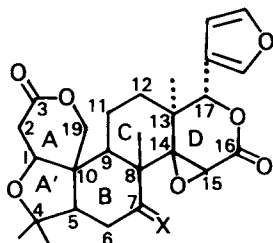
Limonoids are a group of chemically related triterpene derivatives found in the Rutaceae and Meliaceae. Limonin, a bitter member of the group, occurs widely in citrus juices. It has commercial significance because bitterness (excessive bitterness in the case of grapefruit) reduces juice quality. Dreyer (1) and Connolly et al. (2) have reviewed the chemistry and biochemistry of limonoids. More recently Maier et al. (3) published a comprehensive review of the limonoid constituents of Citrus and the impact of limonin bitterness on juice quality. This paper summarizes the chemical, biochemical and juice quality aspects of limonoids in Citrus (and related genera) and presents relevant advances since previous reviews.

Limonoid Structures and Relevant Chemistry

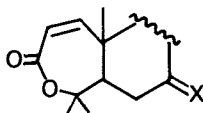
Limonin (I) is a highly oxygenated triterpene derivative whose structural features include a furan ring, two lactone rings, a five-membered ether ring, and an epoxide. All other citrus limonoids also contain the furan ring and at least one of the lactone rings. Compounds I, III, V-XII, and XIV-XVI were known as natural citrus constituents at the time of our last review (3). Since then several other citrus limonoids have been isolated and their structures determined: limonol (II), obacunol (IV), and deoxylimonol (XVII) from grapefruit seeds (4), isolimononic acid (XIII) from sour orange and grapefruit seeds (5), deoxylimononic acid (XVIII) from grapefruit seeds (6), and methyl deacetylnominate (VIII), calamin (XIX), retrocalamin (XX), cyclocalamin (XXI), and methyl isoobacunoate diosphenol (XXII) from calamondin seeds (7).

Table I (8) shows the concentrations of limonoids in seeds of several citrus species, as well as the relative proportions of neutral and acidic limonoids in each. Tables II and III (8) show the relative amounts of the major individual neutral and acidic limonoids, respectively, in several species. The cases in which two different samples of the same species were analyzed show the

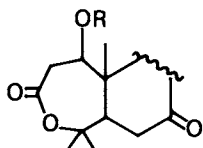
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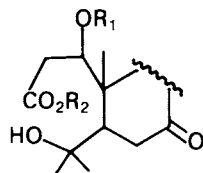
- I LIMONIN $X=O$
 II LIMONOL $X=\alpha-OH, \beta-H$



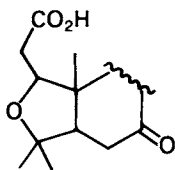
- III OBACUNONE $X=O$
 IV OBACUNOL $X=\alpha-OH, \beta-H$



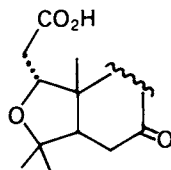
- V DEACETYLNOMILIN $R=H$
 VI NOMILIN $R=Ac$



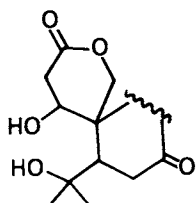
- VII DEACETYLNOMILINIC ACID
 $R_1=R_2=H$
 VIII METHYL DEACETYLNOMILINATE
 $R_1=H, R_2=Me$
 IX NOMILINIC ACID $R_1=Ac, R_2=H$



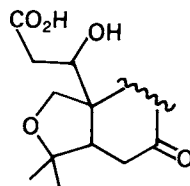
X ISOOBACUNOIC ACID



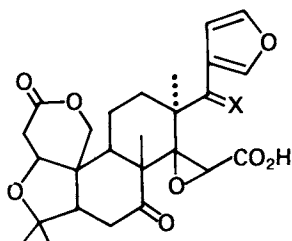
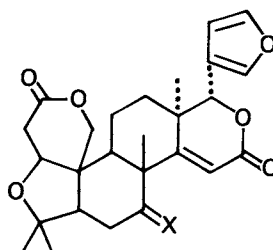
XI EPIISOOBACUNOIC ACID



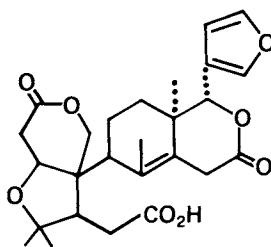
XII ICHANGIN



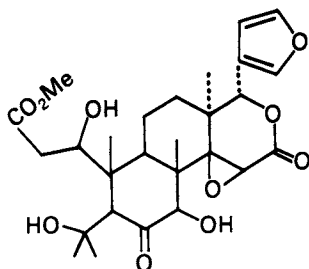
XIII ISOLIMONIC ACID

XIV LIMONOIC ACID A-RING
LACTONE X=H, OHXV 17-DEHYDROLIMONOIC
ACID A-RING LACTONE X=O

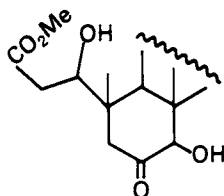
XVI DEOXYLIMONIN X=O

XVII DEOXYLIMONOL
X= α -OH, β -H

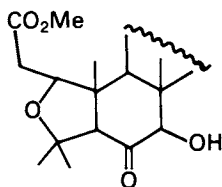
XVIII DEOXYLIMONIC ACID



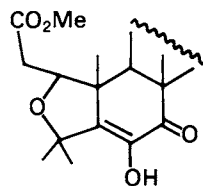
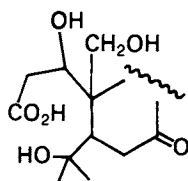
XIX CALAMIN



XX RETROCALAMIN



XXI CYCLOCALAMIN

XXII METHYL ISOOBACUNOATE
DIOSPHENOL

XXIII 19-HYDROXYDEACETYLNOMILINIC ACID

Table I. Concentrations of Limonoids in Citrus Seeds (8)

Seeds	Total, % of fresh weight	% of total limonoids	
		Neutral	Acidic
Grapefruit	1.5	77	23
Valencia Orange	1.1	84	16
Lemon	1.1	61	39
Calamondin	0.75	83	17
Kumquat	0.61	55	45

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ranges of variation in composition which can be expected.

The chemical reactions of limonin have been reviewed previously (1,3). From a practical standpoint the most important reactions are the opening and closing of the lactone rings. In citrus leaf and fruit tissue the naturally occurring form is a salt of limonoic acid A-ring lactone (XIV), in which the A-ring is closed and the D-ring is open. This tasteless compound is only stable in the salt form. At acidic pH's, and more rapidly in the presence of citrus limonoate D-ring lactone hydrolase, the D-ring closes to form the bitter substance limonin (9). The gradual conversion of XIV to limonin occurs whenever fruit tissues are disrupted, such as, through severe bruising, freeze damage or during juice expression (10). Limonin occurs as such, with both lactone rings closed, in citrus seeds. The seeds also contain small amounts of the salt of XIV (10,11).

Structural relationships and pathways

The citrus limonoids may be divided into groups on the basis of structural similarities. One such criterion is the nature of C-19, which may be either methyl, as in obacunone (III), or oxy-methylene, as in limonin. Another characteristic feature is the five-membered ether (A') ring in compounds such as limonin and isoobacunoic acid (X). The calamondin limonoids XIX-XXII are unique in two respects: the 3-carboxyl is methylated and C-6 is oxygenated. While these structural groupings are helpful in suggesting possible biosynthetic pathways, they do not allow a decision to be made as to the actual pathway by which limonin is synthesized. For instance, X contains all of the structural features of limonin except for 19-oxygenation and lactonization and thus could be considered the immediate precursor. However, ichangin (XII) is also a possible immediate precursor, containing the A lactone ring but lacking the A'-ring. The true pathway will ultimately have to be determined by incorporation studies of possible precursors in radioactive form. Nevertheless, it seems highly probable that deacetylnomilinic acid (VII) is a key limonoid intermediate. Lactone ring closure and/or acetylation

Table II. Relative Concentrations of Neutral Limonoids in Citrus Seeds (8)

<u>Seeds</u>	<u>% Distribution</u>			
	<u>Limnin</u>	<u>Nomilin</u>	<u>Obacunone</u>	<u>Deacetylnomilin</u>
Grapefruit	76	15	1	8
	83	11	5	1
Valencia Orange	50	34	1	15
	75	10	1	5
Navel Orange	87	10	1	2
Sour Orange ^a	37	26	1	11
	39	21	1	17
Lemon	39	31	29	1
	40	40	20	1
Lime	70	28	1	1
Tangelo	43	43	13	1
Tangerine	63	16	1	20
	60	18	1	21

^aAlso Ichangin 25
22

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Table III. Relative Concentrations of Acidic Limonoids in Citrus Seeds (8)

<u>Seeds</u>	<u>Nomilinic Acid</u>	<u>Deacetylnomilinic Acid</u>	<u>Isolimononic Acid</u>
Grapefruit	86 85	5 13	8 1
Valencia Orange	83	17	-
Sour Orange	6 3	47 64	47 32
Lemon	95	5	-
Lime	76	15	9
Tangelo	56	44	-
Tangerine	33	33	34
Navel Orange ^a	90	10	-

^aFruit tissues

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reactions could convert it to deacetylnomilin (V), nomilinic acid (IX), or nomilin (VI), and an elimination reaction could then produce obacunone (III) from either V or VI. A 1,4-cyclization of VII would lead to X, which could form limonin by 19-hydroxylation and lactone ring closure. 19-Hydroxylation of VII would form XXIII, which could undergo 4,19-cyclization to produce isolimoninic acid (XIII) or 1,4-cyclization, followed by lactone ring closure to limonin. Alternatively, XXIII could form XII by lactone ring closure and the latter could then cyclize to produce limonin.

The biosynthetic pathway of the calamondin limonoids seems straightforward. Methyl deacetylnomilate (VIII) is an obvious precursor of calamin (XIX), and the latter could then be converted to retrocalamin (XX) by a retroaldol-type reaction. 1,4-Cyclization of XIX would produce cyclocalamin (XXI), which could form methyl isoobacunoate diosphenol (XXII) by oxidation of the 7-hydroxyl to a ketone, followed by enolization.

Biosynthesis

Since this subject was reviewed in 1977 (3) important progress has been made in studies of the biosynthesis of limonoids in Citrus. Analyses of Citrus for XIV content at various maturity stages and radioactive tracer work showed that limonoids are actively synthesized in citrus leaves, particularly in young leaves (12). Hasegawa et al. (13) attempted to locate limonoids within mesophyll cells and found that limonoids are present in chloroplasts. Further fractionation of this organelle showed the presence of limonoids in grana. The grana isolated from lemon leaves contained as much as 1,000 ppm of XIV. These findings suggested that limonoids are most likely synthesized in chloroplasts, although further work is needed to confirm this point.

When 5-g-size navel oranges were fed with 44×10^6 cpm of acetate-2- ^{14}C at the rate of 4.4×10^6 cpm per day for 10 consecutive days either by injection of the fruit or by application on the peel, no labeled XIV was detected in the fruit. Also, no ^{14}C was incorporated into XIV when 5-g-size navel orange was similarly fed with 20×10^6 cpm of mevalonate-2- ^{14}C . When labeled acetate was fed to leaves adjacent to a 5-g-navel orange, radioactivity was incorporated into XIV in the fruit (12). These results suggest that limonoids are most likely not synthesized in the fruit tissues (12). However, they do not rule out the possibility that a precursor(s) of limonoids beyond acetate or mevalonate is synthesized in leaves and translocated to fruit and there converted to limonoids.

Citrus trees are capable of translocating limonoids from leaves to fruit tissues (12). When 20,000 cpm of XIV, randomly labeled, was fed to a leaf adjacent to a 5-g-size lemon, about 13.3% of the total activity was translocated to the fruit during 20 hrs. These findings of Hasegawa et al. (12,13) show that limonoids in citrus fruit tissues are synthesized in leaves and translocated to the fruit.

It has been mentioned in the previous section that citrus seeds contain high concentrations of limonoids. Datta and Nicholas (14) showed the presence of limonoid biosynthetic systems in germinated Valencia orange seeds by demonstrating the incorporation of mevalonate-2- ^{14}C into I. However, Hasegawa et al. (8) could not find such systems in lemon seeds, immature or mature. Since limonoids are actively synthesized in young, immature leaves (12), most likely the radioactive I found by Datta and Nicholas was synthesized in the coleoptiles of the germinated orange seeds.

The capability of *Citrus* to translocate limonoids from the fruit tissues to the seeds was demonstrated by administering 1×10^6 cpm of methyl- ^{14}C deacetylnomilate to the stem end of detached calamondin fruits. After 16 hrs of incubation, 7,500 cpm of activity were translocated to the seeds, and over 90% of the total activity in the seed extract was recovered as the original substrate (8). These results show that limonoids present in citrus seeds are translocated through the fruit tissue.

Inhibition of Biosynthesis. Triethylamine derivatives such as 2-(4-ethylphenoxy)triethylamine and 2-(3,4-dimethylphenoxy)-triethylamine markedly inhibit the accumulation of limonoids in citrus leaves (15). For example, young lemon leaves sprayed with 500 ppm of 2-(4-ethylphenoxy)triethylamine contained only 27 ppm of XIV 8 days after the treatment, whereas the control contained 344 ppm. Similarly, those sprayed with 300 ppm of the compound contained 0.3 times as much XIV as the control.

Metabolism

Metabolism in Bacteria. Three species of bacteria, which are capable of metabolizing limonoids, have been isolated from soil by Hasegawa et al. (16,17,18,19), Table IV.

Table IV. Limonoid-metabolizing Bacteria and Metabolic Pathways

<u>Bacteria</u>	<u>Major Metabolites</u> ^a	<u>Pathways</u>
<u>Arthrobacter globiformis</u>	17-dehydrolimonoate	17-dehydrolimonoid
<u>Pseudomonas</u> sp. 321-18	deoxylimonate deoxylimonin	deoxylimonoid 17-dehydrolimonoid
No. 342-152-1	17-dehydrolimonoate deoxylimonate deoxylimonin	17-dehydrolimonoid deoxylimonoid

^aWhen grown on limonoate

Two metabolic pathways of limonoids have been established: one via 17-dehydrolimonoids and the other via deoxylimonoids. *A. globiformis* metabolizes limonoids via only the 17-dehydrolimonoid pathway (17). *Pseudomonas* sp. 321-18, on the other hand, produces in its growth media only XVI and XVIII, which are metabolites of the deoxylimonoid pathway, but this organism also possesses the 17-dehydrolimonoid pathway (18). This organism undoubtedly metabolizes limonoids preferentially via the deoxylimonoid pathway. Bacterium No. 342-152-1 produces metabolites involved in both pathways (19). The ratio of XV to XVIII is about 3 to 1, suggesting that this organism metabolizes limonoids preferentially via the 17-dehydrolimonoid pathway.

Enzymes in Bacteria. Limonoate dehydrogenases, which catalyze the conversion of XIV to XV, have been isolated from three species of bacteria by Hasegawa et al. (17,18,19). Each dehydrogenase has different characteristics (Table V).

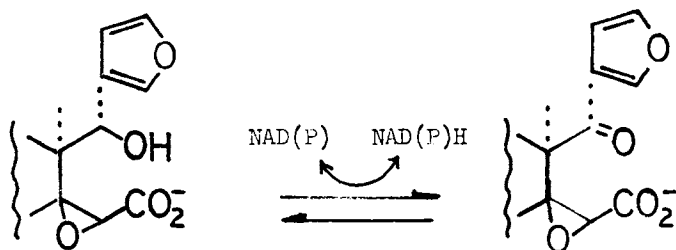


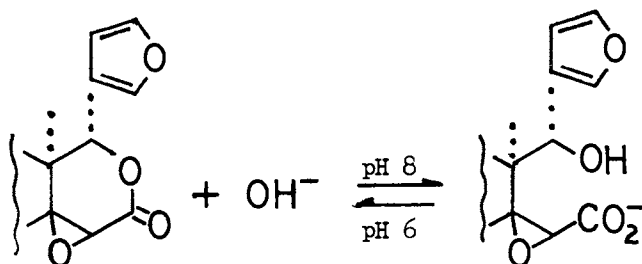
Table V. Bacterial Limonoate Dehydrogenases

<u>Bacteria</u>	<u>Cofactors</u>	<u>Optimum pH</u>
<u>Arthrobacter globiformis</u>	NAD	9.5
<u>Pseudomonas</u> sp. 321-18	NAD or NADP	8.5
No. 342-152-1	NAD	7.5

These enzymes attack not only XIV, but other limonoids which contain a furan ring and epoxide, provided that the D-ring is open. The enzymes require sulfhydryl groups and Zn^{++} for their catalytic activity.

Deoxylimonin hydrolase, which catalyzes the conversion of XVI to XVIII, has been isolated from cell-free extracts of *Pseudomonas* sp. 321-18 (20). This enzyme attacks only the closed D-ring of XVI and requires no cofactor for its catalytic activity. Unlike other common hydrolases, this enzyme is inhibited by *p*-chloromercuribenzoate and HgCl_2 .

Limonin D-ring lactone hydrolase has been isolated from



Pseudomonas sp. 321-18 (21). Hydrolyzing activity is optimal at pH 8.0, whereas lactonizing activity is optimal at pH 6.0. The enzyme attacks limonoids whose structures differ from I in the vicinity of the A or A'-ring. The enzyme does not attack XVI and XVIII.

Metabolism in Citrus. Recently, Hasegawa et al. (6) have established the presence of a deoxylimonoid pathway in Citrus. Methyl- ^{14}C VIII was metabolized in leaves of calamondin to form a deoxy derivative, showing the presence of epoxidase activity which is required for the first step of the deoxylimonoid pathway. Compound XVIII, the product of the second step, was also isolated from grapefruit seeds (6). Furthermore, deoxylimonate A-ring lactone hydrolase, which is involved in the third step of the pathway, was also detected in grapefruit seeds (6). These findings clearly show that limonoids are metabolized in Citrus not only via the 17-dehydrolimonoid pathway as previously established (22), but also via the deoxylimonoid pathway.

Limonin D-ring lactone hydrolase is the only limonoid enzyme which has been isolated from Citrus and characterized (11). It is of interest to note that this enzyme is extremely heat resistant. It requires 15 min of heating at 100°C to inactivate it completely. Its functional characteristics are very similar to those of the bacterial hydrolase mentioned previously.

Activities of limonoate dehydrogenase (22), epoxidase (6) and deoxylimonate A-ring lactone hydrolase (6) have been demonstrated in Citrus, but they have not been isolated yet.

Organoleptic Aspects

Limonic has been known to be an intensely bitter substance since it was first isolated in 1841 by Bernay (23). Beginning in 1966 the development of analytical methods for limonic allowed correlations of apparent bitterness with juice limonic content to be undertaken. The various reports of relative bitterness of suprathreshold limonic levels have been reviewed by Maier et al. (3). The general conclusions reached in these tests were that limonic levels less than about 6 ppm were generally nonbitter and that the bitterness perceived at higher limonic levels varied with the sweetness, acidity and oil levels of the juice.

Individual vs. Group Bitterness Thresholds. A comprehensive study of limonic thresholds in model systems and in orange juice was reported by Guadagni et al. (24). Limonic thresholds, the minimum concentration perceived by human subjects, were determined under closely controlled conditions by a screened panel. A panel of 27 judges, chosen from a group of 60 for their individual consistency in detecting bitterness, showed a wide range of sensitivity. The most sensitive individual had a limonic threshold in orange juice of 0.5 ppm while that of the least sensitive was 32 ppm (Table VI). Thirty percent of the panel could detect 2 ppm limonic and 62% could detect 4 ppm. The group threshold for this test was 6 ppm in an orange juice of pH 3.8, Brix/acid ratio (B/A) of 14.8.

Most taste studies concentrate on the group threshold rather than the thresholds of the individuals in the group. The above study demonstrates that both are important. The group threshold

Table VI. Thresholds of Individuals for Limonic Bitterness in Orange Juice^a (3)

<u>Limonic Threshold</u> <u>(ppm)</u>	<u>Cumulative % of Panel</u>
0.5	8
1.0	17
2.0	30
3.0	49
4.0	62
5.0	70
6.0	75
10.0	91
32.0	99.5

^a pH 3.8, B/A 14.8

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is useful to determine the generalized effect of a particular taste factor. However, individual thresholds are helpful in studies aimed at determining the taste quality parameters of juice acceptable to a wider segment of the population, especially those individuals most sensitive to bitterness.

Influence of Juice Components on Bitterness. The effects of a number of juice variables were also studied (24). A pH optimum for the limonin group threshold was observed at pH 3.7 to 3.9 in reconstituted orange juice concentrate systems. The threshold was a maximum of 6.4 ppm at pH 3.8 with a threshold of 3.4 ppm at pH 3.5 and pH 4.1. Varying the pH while keeping the B/A constant again showed a maximum threshold of 6.5 ppm at pH 3.8. At a constant pH of 3.65, increasing the B/A from 10 to 16 increased the limonin threshold from 6.2 to 8.5 ppm. A higher B/A had no greater effect. When judges were presented juices containing 4 ppm limonin with different pH and B/A values, they indicated a definite preference for those juices whose pH and B/A levels resulted in thresholds above 4 ppm as predicted from the above data. This confirmed the correlation between high limonin threshold (low limonin detection) and taste preference in orange juice.

Another important interaction is that of limonin with the bitter flavanone glycoside naringin. Both of these bitter substances are present in grapefruit juice and Guadagni et al. (25) found that they interact at subthreshold levels in an additive way. Less than threshold amounts of limonin or naringin contribute to the bitterness of a mixture of the two compounds. The bitterness of the mixture can be predicted by adding the taste-unit contribution of each component (taste unit = concentration/threshold).

Influence of Sweeteners on Bitterness. In model system studies, natural fruit juice sugars were observed to raise the limonin threshold (24). An expanded study of natural and artificial sweeteners (26) demonstrated that sucrose, neohesperidin dihydrochalcone (NHD), hesperetin dihydrochalcone glucoside (HDG) and aspartylphenylalanine methyl ester (AP) all raise the limonin threshold. At low sweetness levels HDG was the most effective followed by AP and NHD. Sucrose was without effect up to the 2% level. At sweetness levels equivalent to 1% sucrose, HDG, AP and NHD raised the limonin threshold in water from 1.0 ppm to 3.2, 2.5 and 1.3 ppm, respectively. Because of its high sweetness intensity, the concentration of NHD (16 ppm) was considerably lower than HDG (80 ppm) and AP (90 ppm). At 3-10% sucrose sweetness equivalency, the effectiveness of NHD increased substantially, sucrose moderately and HDG slightly, while that of AP decreased. Therefore, the sweeteners HDG, AP and NHD can effectively suppress limonin bitterness at low concentrations.

NHD has also been found to suppress naringin bitterness (26). It was suggested as being especially useful in upgrading the flavor of low B/A, early-season grapefruit juice (27).

Bitterness Suppressors. Limonin bitterness suppression has also been reported (28) to occur when the citrus constituent neodiosmin (NEO), the tasteless flavone analog of the bitter flavanone neohesperidin (29), is present in solutions or juices containing limonin. Low levels of NEO were effective in raising the limonin threshold in orange juice, reducing bitterness of supra-threshold levels of limonin, and even reducing the bitterness of very high levels of limonin. NEO is also an effective suppressor of naringin bitterness and would, therefore, be especially useful in bitter grapefruit juice which contains both naringin and limonin. On the basis of the taste tests a process for reducing the bitterness of citrus juices was proposed which comprises adding 50 to 150 ppm neodiosmin (30). Another tasteless flavone glycoside that is present in grapefruit and bitter orange, rhoifolin, has the ability to partially suppress the bitterness of naringin (29).

Preharvest, Postharvest and Processing Variables. These factors have a substantial influence on the limonin content of the juice. A detailed discussion of these influences is given by Maier et al. (3). Preharvest factors such as species and cultivar, rootstock, fertilization and irrigation practices, and time of harvest all have an important bearing on the limonoid content of the fruit. During the postharvest period, limonoid metabolism gradually reduces the XIV content of the fruit tissues. The extent of XIV loss increases with holding time and temperature. The metabolism is accelerated by brief exposure to ethylene gas or to a dilute solution of ethylene generating substances such as 2-chloroethylphosphonic acid (31).

Commercial navel orange, Shamouti orange and grapefruit juices, and laboratory samples of Murcott orange and Natsudaikai juices from early season fruit contain high levels of limonin (3). Under certain conditions limonin bitterness can be found in essentially all types of citrus juices. The rootstock has been shown to be an important factor in determining the time required after commercial maturity is reached for the fruit to yield juice with low limonin levels. The rootstocks promoting fastest attainment of low limonin levels are trifoliate orange, tangelo and Cleopatra mandarin, with sweet orange being intermediate, and rough lemon, sweet lime, Kusaie lime and East India lime retarding the disappearance of limonin (32).

The amount of limonin incorporated into the juice is influenced by a number of juice processing variables. Factors such as the maceration of albedo, central vascular bundle and carpillary membranes, the time of contact between the rag and the juice, and the amount of pulp incorporated into the final juice product are known to influence juice limonin content. The uneven distribution of XIV in the various tissues of the fruit (Table VII) is largely responsible for the effects of processing variables (33).

Table VII. Limonoid Distribution in Desert Navel Orange Tissue^a

	<u>Limonoid A-Ring Lactone Content^b</u>	
	<u>ppm fresh weight</u>	<u>mg per fruit</u>
Albedo and Flavedo	415	22
Carpellary Membranes	305	10
Juice Vesicles	15	1.9

^aHarvested November 12^bDetermined as limonin

Only about 6% of the total XIV content of the fruit is present in the juice vesicles. Any step in juice preparation that increases incorporation of the other tissues into the juice or that increases the extraction of XIV from those tissues will increase the limonin content of the juice. Consequently, techniques that increase juice yield, such as hard versus soft extraction, tend to increase limonin content (34).

Limonin Levels of Citrus Juices. A compilation of limonin values in various citrus juices is given in (3). Limonin has been found in essentially all varieties of citrus juices examined. Although there is a considerable range of limonin values reported, certain trends are apparent.

Limonin levels generally decrease as the season progresses. This decrease with maturity of the fruit is seen in most varieties tested, although the magnitude of the change and the level at any one time of the year or any part of the harvesting season is variable.

Two extensive studies of limonin content throughout the season were recently conducted by Albach et al. (35). Commercial orange and grapefruit juices from three citrus processing plants in south Texas were sampled twice each day at three-week intervals from early November to late June for two consecutive years. The average limonin content of these juices is summarized in Table VIII. The November orange juice samples were all above the group limonin threshold determined by Guadagni (24). Some of the samples obtained through February were also above the 6 ppm limonin group threshold. Throughout the season a significant number of juices contained enough limonin for bitterness to be detectable by 30-50% of the individuals of Guadagni's taste panel. The limonin content of these orange juices would, therefore, be likely to lower the taste quality of the juice for a substantial portion of the population.

Another set of tests of two orange varieties from 5 locations in Texas were sampled at two-week intervals throughout their season of maturity and juiced in the laboratory on a commercial test

Table VIII. Limonin Content of Commercial Orange and Grapefruit Juices Throughout the Harvesting Season

Processing Date	Limonin Content (ppm)			
	Orange		Grapefruit	
	Range	Average	Range	Average
November	6.2-8.2	7.2	11.2-12.2	11.4
December	4.2-7.8	5.3	7.1-11.6	10.0
January	3.3-5.5	4.5	7.4-16.6	9.1
February	2.3-6.8	3.8	5.3-9.7	7.1
March	2.5-3.9	3.3	4.4-7.1	5.1
April	2.1-3.8	2.7	2.2-4.7	3.8
May	1.7-2.1	1.9	2.4-3.8	3.1
June	1.8-2.4	2.1	---	2.1

Source: (35)

extractor (36). Again the limonin levels decreased as the season progressed for both Hamlin and Marrs early oranges on Texas sour orange rootstock. The Marrs variety was suspected of having a bitterness problem because it arose as a bud sport from the Washington navel. The data show little consistent difference in the limonin levels of the two varieties in any one grove. Much more significant differences were seen in fruit of the same variety from the different grove locations, probably due to differences in cultural practices, soil type and environmental factors.

While most of the limonoids of *Citrus* have been isolated from seeds, several occur in detectable amounts in other parts of the fruit. Minor amounts of deacetylnomilin, nomilin, obacunone, deacetylnomilinic acid and nomilinic acid were identified in extracts of navel orange peel (37). 17-Dehydrolimonoate A-ring lactone was isolated from peel and juice of navel oranges (38) and nomilin has been reported to occur in grapefruit juice and juice vesicles (39). The only *Citrus* limonoids known to be bitter are limonin, VI, XII, obacunoic acid and IX (3).

Influence of Limonin Content on Juice Quality. Several studies have shown that limonin bitterness detracts from juice quality. The taste tests reported by Guadagni (24) using screened laboratory personnel indicated that factors which raise the limonin threshold in orange juice have a beneficial effect on preference. Fellers (40) reported results of taste tests of grapefruit juice with added limonin and naringin given to a Washington D. C. consumer-type taste panel of 72 persons. These tests showed a highly significant linear drop in preference ratings with

successively higher levels of bitterness within each B/A group. The inverse relationship between limonin content and taste preference was confirmed in another study (41) using a stepwise multiple regression analysis of data from 60 samples of commercial frozen-concentrated orange juice (FCOJ) packed during two seasons. This and a latter report (42) concluded that limonin content was highly correlated with the flavor quality of the juice.

Juice Quality Standards. In recent years limonin content has become widely recognized as a quality factor in most citrus juices. Previously limonin had been considered to be important in the juice quality of only a few high limonin cultivars. Limonin is being used routinely by some processors as a quality control element. In addition, limonin content is now included in the Florida State Grades for canned and chilled grapefruit juice and frozen concentrated grapefruit juice (43). Grade "A" juice packed during the period from August 1 to December 1 must contain less than 5 ppm limonin or less than 600 ppm naringin (Davis test method). Grade "B" juice must contain less than 7 ppm limonin or less than 750 ppm naringin.

Determination of Limonin Content. A summary of the methods reported through 1976 for the quantitative determination of limonin in citrus juices was published previously (3). Since then improvements have been made in the high-pressure liquid chromatographic method (44, 45) and advances have been made in the development of enzymatic (46) and immunoassay (47, 48) (see Chapter 15) methods. The latter methods hold great promise for the future. However, in each case the required enzyme or antibody is not yet commercially available. Thus, while substantial progress is being made the need still exists for a simple, sensitive, and rapid commercially available method for routine industrial quality control purposes.

Control of Juice Bitterness. A number of advances have been reported in this field since it was last reviewed (3). A commercial application of the cellulose acetate adsorption technique for the removal of limonin from citrus juices was undertaken (49). New sorbent gel forms of cellulose esters for adsorption of limonin were developed (50). Knowledge was gained that limonoids are biosynthesized in citrus leaves and translocated to the fruit (12) and that specific bioregulators can inhibit accumulation of XIV in citrus leaves (15). Additional studies were carried out on the use of neodiosmin to suppress limonin and other types of bitterness (30, 51). The influence of extractor and finisher pressures on the level of limonin and naringin in grapefruit juice was reported (34). Also, further studies were conducted on the microbial sources and properties of limonoate dehydrogenase (52), the enzyme that converts XIV to XV and can be used to prevent limonin from forming in freshly expressed citrus juices (53).

As these new techniques for reducing limonin bitterness are developed and come into use, they will afford additional and more direct means of achieving higher quality citrus juices. This will be especially important for that significant portion of the population that has a limonin bitterness threshold in the vicinity of and below 2 ppm. Until then, much can be done to reduce bitterness by careful management of factors influencing juice limonin levels (choice of cultivar, rootstock, ripeness, postharvest metabolism, juice extraction parameters, pulp contact time, blending of juice, etc.) and factors influencing the perception of bitterness (juice pH, acidity, sweetness, etc.).

Abstract

Limonoids are a group of chemically related triterpene derivatives found in the Rutaceae and Meliaceae families. Limonin is an intensely bitter limonoid that occurs widely in citrus juices where its presence at above threshold levels generally detracts from juice quality. Studies have shown that the metabolically active form of limonin is limonoate A-ring lactone (LARL) which is nonbitter. LARL is synthesized in the leaves and translocated to the fruit and seeds. It is slowly degraded in the fruit to non-bitter products by at least two metabolic pathways. LARL undergoes acid catalyzed lactonization to limonin when the fruit tissues are disrupted in juice preparation. Group bitterness thresholds for limonin in orange juice have been reported to be in the 6 ppm range. On the other hand, individual thresholds as low as 0.5 ppm have been reported. In the latter study, 30% of the panel had thresholds of 2 ppm or below. While limonin content tends to be high in the juice from early-season navel, Shamouti, and Murcott oranges, available data indicate that levels of 2 ppm are not uncommon in commercial orange juice. Bitterness is modulated by juice properties including soluble solids, citric acid content and pH. In addition, several tasteless citrus flavonoids specifically suppress bitterness and increase juice acceptability, as do the flavanone glycoside derived dihydrochalcone sweeteners. The above areas are reviewed in detail and new developments in limonoid biochemistry are discussed.

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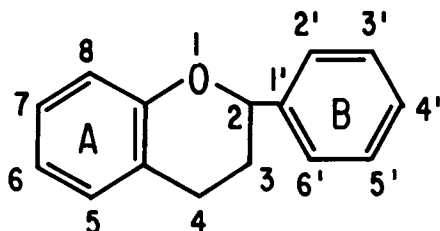
Flavonoids and Citrus Quality

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Flavonoids are one of the most widely distributed and diverse chemical groups in the plant kingdom. While flavonoids can be found in nature in many organisms, from bacteria to higher plants, they are most prevalent in the higher plants. They have been found in the roots, stems, flowers, pollen, fruit, seeds, wood and bark.

In terms of chemical structure, flavonoids are C_{15} compounds arranged $C_6-C_3-C_6$ with the central group usually linked with oxygen and numbered as shown below:



These compounds are differentiated primarily by the oxidation state of the central three carbon atom unit. Thus, as shown in Figure 1, some compounds are classified as flavones, flavanones, flavonols, anthocyanins, etc. (The A and B rings have been left off for clarity). A secondary means of differentiating flavonoids is by the position and numbers of attached hydroxy, methoxy or sugar units. In citrus, flavonoids usually occur as glycosides, although the polymethoxylated flavones are a notable exception.

Metabolically flavonoids can be thought of as a combination of two distinct units: 1) the C_6 fragment of the A ring and 2) the C_3-C_6 fragment of the B ring. However, while significant progress has been made in the areas of biological origins and interrelationships of flavonoid compounds the exact biological function of these compounds remains a mystery.

Quality in any food product is usually defined in terms of a

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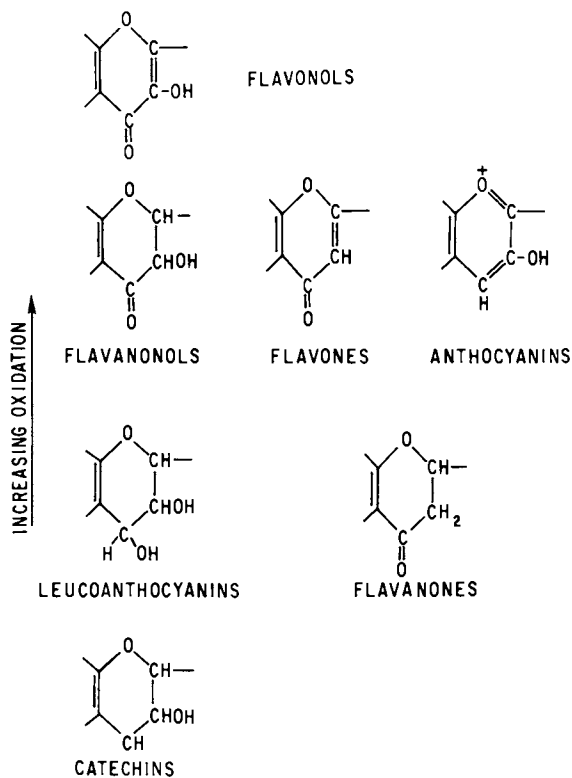


Figure 1. Flavonoid structures and nomenclature as determined by the central C_3 group (A and B rings have been left off)

number of factors. These factors are usually evaluated by the human senses, namely, sight, taste, touch and smell. The primary sense in evaluating a food item is taste, although other senses, such as sight and smell, can influence the perception of taste. Flavonoids play a major role in the determination of citrus quality primarily due to the taste of flavanone neohesperidosides. Flavonoids do not play a significant role in the visual determination of fruit or juice quality. Although many flavonoids in the plant kingdom are highly colored, most citrus flavonoids are colorless. The compounds responsible for the deep yellows and oranges in citrus are carotenoids. Flavonoids play a significant role in human nutrition but since this area is the subject of chapter 3, the present discussion will be limited to how flavonoids affect citrus quality.

Physical and Chemical Characteristics

Chemical and physical properties of individual citrus flavonoids and flavonoids in general have been discussed in other reviews (1-5). In the flavonoid molecule the A and B rings are stable and relatively unreactive. The central C₃ group is the most reactive portion of the molecule, especially if linked with oxygen to form a heterocyclic ring. The dihydropyrone ring of flavanones is very reactive and readily undergoes ring opening reactions with base or acetic anhydride (3). However, flavonoids are generally stable under normal processing or storage conditions. Flavonoid physical properties, such as UV absorption spectrum and solubility are highly dependent on the bonding and arrangement of the atoms in the C₃ group. Significant shifts are observed in the absorption maxima from the UV spectra of flavonoids when ring opening or complexing reagents are introduced. Thus, much structural information can be inferred from the spectral changes of these compounds (6). There is little enzymatic activity to alter the flavonoid composition during storage as the freshly extracted juice is pasteurized to inactivate most enzymes.

Hesperidin Solubility. Hesperidin, a tasteless flavanone glycoside, is the least soluble of all citrus flavonoids. It is found in practically every variety of citrus (5) and is the major flavonoid in sweet oranges and lemons. In fruit or leaves, hesperidin is found as a soluble complex which can be extracted with water or alcohol (5). During juice extraction, the complex is destroyed and hesperidin slowly precipitates as fine, white, needle-shaped crystals. Once in the solid form, hesperidin can be redissolved in formamide, pyridine or in dilute alkali.

Hesperidin crystals are found in frost damaged oranges (8) where cells have been disrupted due to the formation of ice crystals. It occasionally precipitates out of concentrated orange juice products during storage and is often found as a

thin crust on the surfaces of falling film evaporators used in the production of frozen orange concentrate (9). Hesperidin crystals have also been found to coat the surface of juice extractors. This crust reduces the rate of evaporator heat exchange which adds to the energy costs and slows the rate of concentrate production. If the evaporator surfaces are not cleaned periodically, hesperidin crystals will flake off into the concentrated juice. Juice containing excessive amounts of hesperidin crystals is downgraded (10). Shown in Figure 2 are acceptable and unacceptable amounts of hesperidin crystals from 710 ml of reconstituted orange juice. It should be pointed out that the presence of hesperidin crystals in a citrus product is a visual defect and does not affect the flavor of the product.

Naringin Solubility. Naringin, the major flavonoid in grapefruit and pummelo, has been observed (11) to crystallize in canned fruit or juice during storage. This problem was found primarily when slightly immature fruit was used. Since naringin concentrations are highest in immature fruit and generally decrease as the fruit matures, the easiest way to prevent this precipitation problem is to use mature fruit. Another means of reducing the naringin content is to reduce or eliminate from the final product those portions of the fruit containing high naringin concentrations, i.e., the albedo and segment membrane.

Methods of Analysis

The establishment of precise objective measurements for citrus quality has been hindered by the lack of good analytical methods and the lack of consensus on a common definition of quality. The evaluation of bitterness is a prime example. Ideally the method should be rapid, specific, accurate and inexpensive. Unfortunately, no method has been developed that satisfies all four criteria. Compromises and trade-offs must be made.

One of the earliest methods to measure the bitter naringin and other flavanones in grapefruit juice was developed by W. B. Davis in 1947 (12). This test is based on the reaction of dilute alkali with flavanones to form the corresponding yellow chalcones. The flavanone concentration is then determined by measuring the absorbance of the chalcones at 427 nm. Davis pointed out that the procedure was not specific for any flavanone but could be used to determine the principle flavanones in citrus juice, i.e., naringin in grapefruit juice and hesperidin in orange juice. He suggested that the method might also be suitable for the determination of flavones and flavonols. This method is still widely used to measure naringin in grapefruit juice; albeit it is not specific for naringin, it is a simple, rapid and inexpensive method of analysis. However, since grapefruit contains both bitter and nonbitter flavanone glycosides, Davis values are only a crude approximation of bitterness.

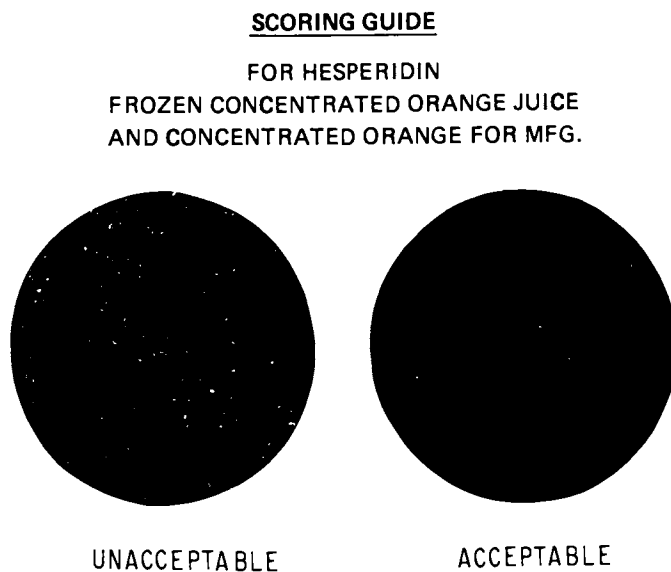


Figure 2. USDA visual aids used to determine acceptable and unacceptable amounts of hesperidin crystals in orange juice

Other colorimetric methods have been developed to determine flavonoid bitterness in citrus products (13, 14, 15, 16). The basic approach involves the addition of a reducing agent, such as sodium borohydride or magnesium, to juice followed by the addition of HCl to form a characteristic color. Again, the color is due to both bitter and nonbitter flavanone glycosides in addition to the tasteless flavanone aglycones. An older and even less specific method is the ferric chloride method (17, 18) for naringin. Unfortunately, this method reacts with many flavonoids, both bitter and nonbitter, as well as citric acid and other hydroxy compounds present in the juice.

Direct ultraviolet spectrophotometric methods have been developed to measure naringin in grapefruit (19) and hesperidin in orange juice (20, 21). While these methods are rapid, they are also nonspecific for flavonoid bitterness.

Chromatographic methods were developed to separate a few of the citrus flavonoids from the complex mixture of citrus flavonoids. The early paper chromatographic methods for flavanones (22, 23) were difficult to quantitate because of band broadening and uneven solvent development. Several thin layer chromatographic (TLC) methods were developed to separate the bitter from the nonbitter flavanone glycosides (24, 25, 26, 27).

Gas-liquid chromatography (GLC) has not been employed for the analysis of flavanone glycosides because they are non-volatile and thermally unstable. One GLC method (28) has been developed for the analysis of the flavanone aglycones. However, the method is extremely time consuming in that the samples must be extracted, hydrolyzed and derivatized before analysis. Furthermore, the procedure cannot distinguish between bitter and nonbitter flavonoids.

Swift (28) developed a TLC-spectrophotometric analysis of five methoxylated flavones from orange peel. Since the analysis consisted of two stages, one for separation and the last for quantitation, the analysis was rather time consuming and also subject to some positive errors due to incomplete sample separation. Tatum *et al.* (29) developed several direct TLC methods for methoxylated and hydroxylated flavones, coumarins and psoralens in citrus. Maier and Metzler (30) developed a semi-quantitative, two-dimensional TLC procedure to determine a number of citrus flavanones, flavanols, flavones, coumarins and psoralen aglycones. High performance liquid chromatographic methods (HPLC) have been developed for the separation of naringin from narirutin (31, 32) in citrus juices. A method to determine the major methoxylated flavones in citrus has been developed (33). Gradient elution HPLC has been employed to separate a wide variety of citrus flavanone glycosides, coumarins and psoralens (34). Thus, with the recent improvements in methodology, it is now possible to evaluate the relative contribution of individual citrus flavonoids because the concentration of each can now be rapidly and accurately measured.

Citrus Flavonoids as Quality Factors

Flavonoids have no odor or mouth feel and, in general, do not contribute significantly to the color of most citrus juices. Their primary effect on citrus quality is due to the bitter taste of certain flavanone glycosides. Thus, quantitative descriptions of desirable citrus qualities are usually based on the absence or maximum concentration limits for these compounds.

Bitterness is a generally undesirable flavor component and is usually detrimental to the quality of citrus products. Any bitterness in orange or tangerine products reduces their quality, whereas, a little bitterness is actually desirable for grapefruit products. However, it has been shown (35) that as the concentration of bitter materials increase, flavor scores and overall product acceptability decrease. Thus, excessive bitterness is usually considered objectionable and the Florida Department of Citrus has enacted regulations which limit the amount of naringin which may be present during the early weeks of the season (36). It is difficult to quantitatively define grapefruit quality in terms of bitterness because individual taste thresholds and bitterness preferences vary markedly. Guadagni *et al.* (37) found that 7% of a 27 member taste panel could detect as little as 1.5 ppm naringin in water. Yet, another 7% of that same panel could not distinguish a 50 ppm naringin solution from water alone. This represents over a 30-fold difference in taste thresholds.

Fellers (38) used a large population of grapefruit juice users to evaluate naringin bitterness preference levels. Some of his data is shown in Table I. Using a low naringin juice he added various amounts of naringin to different lots of the same juice. The tasters were not told the juices contained different bitterness levels but were asked to evaluate each juice on a six point scale from poor = 1 to excellent = 6. As expected flavor scores fell as the naringin content of the juice increased. Correspondingly, the percentage of the panel that thought the juice was too bitter increased with increasing naringin concentration. However, the surprising result of this study was that at 1900 ppm naringin (an excessively bitter juice), 12% of the tasters did not think the juice was bitter enough. These results show the tremendous range in individual bitterness response, as well as in sensitivity.

Taste and Structure of Citrus Flavonoids

Interestingly, flavanone glycosides exist as structural isomers of which one will be intensely bitter while the other is tasteless. The flavanone portion of the bitter molecule is tasteless, while the glycoside portion is tasteless or slightly sweet (1). Bitterness is observed only when the sugars and the

Table I. Evaluation of naringin bitterness as a factor in preference of Florida grapefruit juice

	Naringin conc. (ppm)				
	300	700	1100	1500	1900
Mean rating	3.7	3.6	3.4	3.4	3.3
Bitterness					
Too bitter	17 ^a	31	33	46	51
Just right	59	55	55	44	37
Not enough bitterness	24	14	17	10	12
Don't know	--	--	--	--	--
(Number of respondents)	(201)	(198)	(198)	(197)	(197)

^a Percentage of tasters

Source: (38)

flavanone aglycone are linked in a specific fashion. These compounds have been thoroughly studied to determine the relationship between taste and structure and has been thoroughly reviewed by Horowitz (1, 37). While no single structural feature has been associated with bitterness, the linkage of the sugars rhamnose and glucose is very important. Linked from C-1 in the rhamnose to the C-2 in glucose, the resulting disaccharide is called neohesperidose (2-O-(rhamnopyranosyl) glucopyranose). If the essentially tasteless neohesperidose (40) is linked to a flavanone through the 7-hydroxy position, the resulting flavanone glycoside will be intensely bitter. However, if the same sugars are linked C-1 to C-6 to form 6-O-(rhamnopyranosyl) glucopyranose (rutinose) and linked in the same way to the identical flavanone aglycone, the resulting molecule is tasteless. The different rhamnose-glucose linkages are illustrated in Figure 3 for the bitter naringin and its nonbitter isomer, narirutin.

All citrus flavanone neohesperidosides are bitter and all flavanone rutinoids are tasteless (1). Since the neohesperidose sugar plays such an important role in flavanone bitterness one might expect other flavonoids with a neohesperidose attached at the 7 position to be equally bitter. Fortunately, this is not the case, for even subtle changes in the flavanone molecule can destroy its bitterness. As shown in Figure 4 if the bitter neohesperidin is converted to neodiosmin (the corresponding flavone) the resulting compound is tasteless (41). Similar results have been observed for other flavanone neohesperidosides (1). No citrus flavone glycoside has been found to be bitter. Furthermore, if the rhamnose portion of the neohesperidoside is removed from naringin, the resulting glucoside (prunin) is still bitter, but at a much reduced intensity (See Table II). While

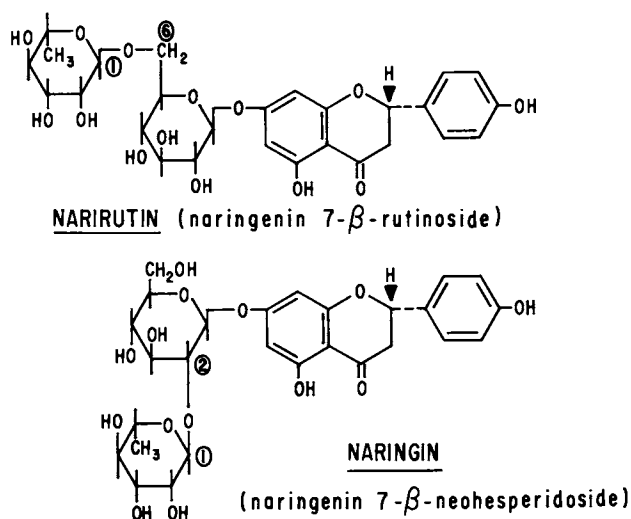


Figure 3. Structural isomers of naringenin illustrating the two possible configurations of the sugars attached at the 7 position. Naringin is bitter whereas narirutin is tasteless.

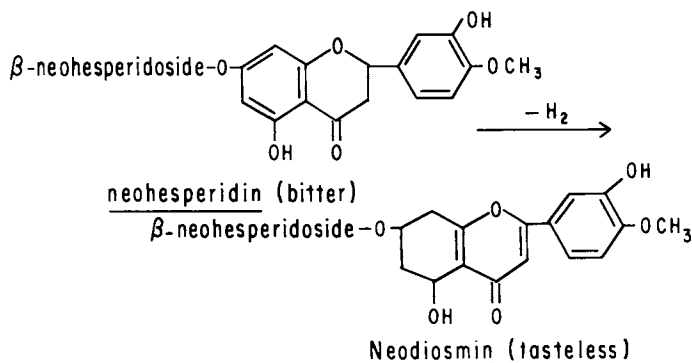


Figure 4. Minor structural changes to the aglycone portion of the molecule can destroy bitterness. Adding a single double bond between Carbons 2 and 3 will convert bitter flavanone neohesperidosides to tasteless flavone neohesperidosides.

all flavanone neohesperidosides are bitter, some are more bitter than others. Thus, as shown in Table II, naringin and poncirin are two of the most bitter flavone glycosides found in citrus. Each is about one-fifth as bitter as quinine dihydrochloride. Neohesperidin and neoeriocitrin are only one-tenth as bitter as the other two flavanone glycosides. Thus, it appears the number and position of ring substitutions affects the degree of bitterness of the overall molecule.

Table II. Relative Bitterness of Citrus Flavonoid Aglycones and Glycosides

<u>Compound</u>	<u>Taste</u>	<u>Relative Bitterness*</u>
Hesperetin ^a	Sl. sweet	--
Eriodictoyl ^a	No taste	--
Naringenin ^a	No taste	--
Hesperidin ^b	No taste	--
Neohesperidin ^c	Bitter	2
Narirutin ^b	No taste	--
Naringin ^c	Bitter	20
Didymidin ^b	No taste	--
Poncirin ^c	Bitter	20
Eriocitrin ^b	No taste	--
Neoeriocitrin ^c	Bitter	2
Prunin ^d	Bitter	6

* On a molar basis with quinine dihydrochloride taken as 100

a Aglycones

b Rutinosides

c Neohesperidosides

d Glucoside

Source: (1, 39)

Hagen et al. (42) determined the relative amounts of all the flavanone glycosides in Texas grapefruit. Their results are shown in Table III. Naringin is the dominant flavonoid in grapefruit and is primarily responsible for the immediate bitter taste in grapefruit. The equally bitter poncirin and the much less bitter neohesperidin are present in relatively small amounts and do not contribute significantly to overall bitterness.

While there are no bitter flavone glycosides in citrus, there are some highly methoxylated flavone aglycones that are reportedly bitter. Swift (28) identified sinensetin, nobiletin, tetra-O-methylscutellarein, 3, 5, 6, 7, 8, 3', 4' heptamethoxyflavone and tangeretin from the bitter fraction of orange peel juice. He later determined the individual and collective taste thresholds of these flavones and compared them to the concentrations found in commercial orange juice (43). Orange juice with an added total of 24 ppm of these flavones could not be differentiated from the orange juice alone. Since the maximum total

concentration found in orange juice over several years was 7 ppm, he concluded that these flavones were not important contributors to the flavor of orange juice.

Table III. Flavanone Glycosides in Texas Canned Grapefruit Juice

Compound	Concentration (ug/ml)
Naringin	306
Naringin rutinoside	124
Neohesperidin	10.5
Hesperidin	9.9
Poncirin	17.0
Isosakuranetin rutinoside	5.3

Source: (42)

Flavonoid Bitterness Suppressors

All known citrus flavone glycosides are odorless and generally tasteless yet they appear to have an important function in reducing the perceived bitterness of flavanone neohesperidosides and limonoids. Horowitz (1) was the first to identify the bitterness suppressing function of these compounds. He found that rhoifolin, the flavone equivalent of the bitter naringin, could partially suppress the bitterness of naringin. Thus, higher naringin concentrations were necessary before bitterness could be detected, if the solution contained large amounts of rhoifolin. Guadagni et al. (41) found neodiosmin, the tasteless flavone analog of bitter neohesperidin, to be a very effective bitterness suppressor. As little as 10 ppm in water increased the bitterness threshold of naringin and limonin, a nonflavonoid bitter compound, 3.5- and 4.0-fold, respectively. Neodiosmin was also effective in reducing perceived bitterness of limonin in orange juice.

It was speculated that these flavone neohesperidosides are so similar in structure that they compete with flavanone neohesperidosides for sites on the bitterness receptors in the mouth. This explanation assumed that these flavones could be adsorbed at taste sites without producing an appreciable taste response of their own. Unfortunately, neither of these bitterness suppressors has been found to occur naturally in the sweet orange (*C. sinensis*) although Nakabayashi (44) found rhoifolin in the sour orange (*C. aurantium*). Dunlap and Wender (45) reported finding rhoifolin in grapefruit extracts but no attempt was made to determine its concentration in juice. Neodiosmin has yet to be found in grapefruit. Thus, it remains to be shown if these compounds play any role in the natural reduction of naringin bitterness as grapefruit matures.

Chalcones and Dihydrochalcones. Chalcones and dihydrochalcones are intensely sweet compounds (39) that are effective in raising the threshold at which the bitterness of naringin and limonin is perceived (46). As illustrated in Figure 5, chalcones are easily formed from flavanone glycosides by the addition of alkali and dihydrochalcones are formed from hydrogenated chalcones. Like the flavanone neohesperidosides, the chalcones and dihydrochalcones vary in the intensity of their taste response. Dihydrochalcones are sweeter than chalcones and of the dihydrochalcones, neohesperidin dihydrochalcone (NHD) is the sweetest (39). It has been estimated that NHD is 1,500 times sweeter than sucrose on an equal weight basis. Thus, it has been proposed that NHD be added to excessively bitter juice to reduce the perceived bitterness and increase the quality of the juice (47). A 1969 Florida Department of Citrus Market Research Report indicated favorable sales and consumer acceptance of artificially sweetened grapefruit juice. Fellers (47) found the average taste threshold of NHD in single-strength grapefruit juice to be about 8 ppm. Neohesperidin dihydrochalcone levels between 8-12 ppm produced a significant sweetening of the grapefruit juice with only a slight but acceptable aftertaste. Higher levels of neohesperidin dihydrochalcone produced juices that were excessively and unacceptably sweet. Thus, NHD employed in the proper amounts could be used to upgrade the flavor of early season grapefruit juice or replace a significant amount of sucrose used in covering syrups in canned or frozen grapefruit sections.

Chalcones have been proposed (48) as a precursor that is enzymatically cyclized to form flavanone glycosides. The enzyme responsible for this stereospecific ring closure disappears or is inactivated as the fruit matures (5). However, no chalcones have been isolated from citrus. Therefore, if chalcones are a part of the metabolic pathway that leads to the formation of flavanone glycosides they probably exist as short lived, unstable intermediates. It appears unlikely that these compounds play a significant role in mitigating bitterness at their natural levels.

Taxonomic Significance of Flavonoids

In terms of its flavonoid composition, citrus is unique within the plant kingdom. Some citrus flavonoids are found nowhere else. Furthermore, the relative flavonoid composition varies with each variety. Thus, the flavonoid composition of citrus juices has been proposed (2) as a measure of juice authenticity.

In most of the plant kingdom, flavanones occur only in small amounts compared to other flavonoids, yet they are the predominant flavonoid in citrus. Citrus flavanones usually occur as glycosides, whereas in other plants, flavanones are seldom found in the glycoside form (1). Four types of glycosides have been found in citrus. They are O-glycosides, C-glycosides, rutosides and neohesperidosides. Kefford (2) was one of the first investigators to recognize that flavanone composition could be used to

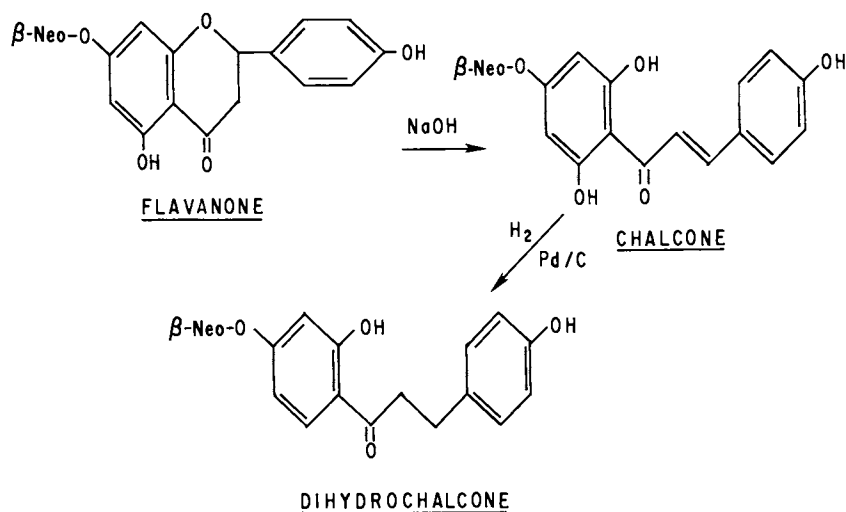


Figure 5. Conversion of bitter flavanone neohesperidosides to the corresponding intensely sweet chalcones and dihydrochalcones

distinguish different varieties of citrus. He classified sweet oranges, mandarins, lemons and citrons together because hesperidin was the predominant flavanone in each. Since the principal flavonoid in grapefruit and pummelo was naringin, Kefferd put these cultivars in a separate group. Horowitz (1) observed that the flavonoid glycosides of many varieties of citrus occurred either all in the rutinoside or neohesperidoside forms. He thus proposed that all citrus could be divided according to their glycoside form.

Albach and Redman (49) confirmed this method of classification with their flavanone survey of 41 citrus cultivars and 49 hybrids from Texas. They found that the relative amounts of flavanones within different cultivars of the same species were fairly constant. Some of their results are shown in Table IV. Most of the commercial citrus cultivars contain only the non-bitter rutinosides, whereas the sour orange and pummelo (*C. grandis*) have only bitter flavanone neohesperidosides. Working with hybrids of known parentage, they found if one variety containing only flavanone rutinosides is crossed with another containing only neohesperidosides, the resulting hybrid contained both types of flavanone glycosides. An example of this is shown in Table IV for the cross between *C. sinensis* x *Poncirus trifoliata*. Thus, it is suspected that the Meyers lemon (*C. limon*) and the grapefruit are hybrids rather than pure varieties because they contain both flavanone rutinosides and neohesperidosides.

Kamiya et al. (50) divided citrus into 12 groups based on the number and kind of flavanone glycosides found. With this classification system, they were able to distinguish between nucellar and zygotic seedlings using both leaves and fruit. Of the 94 hybrids examined, 53 cultivars were judged as true hybrids. The remaining cultivars were not classified as true hybrids because their flavanone glycoside pattern was not sufficiently different from the female parent.

Tatum et al. (29) used the presence of various methoxylated flavones from leaf extracts to distinguish between nucellar and zygotic seedlings. They also identified the predominant flavanone glycoside and three unidentified coumarins in their samples. Ting et al. (51) showed that there were quantitative as well as qualitative differences in the methoxylated flavone content from the juices of different citrus varieties. They further showed that certain blends of juices could be distinguished based on the amounts of methoxylated flavones found.

Both directly and indirectly, flavonoid composition affects the quality of individual citrus cultivars and hybrids. Some cultivars, such as the sour orange, are directly affected by the presence of bitter neohesperidosides to the point they are unpalatable. The quality of other cultivars may be indirectly affected due to the presence of bitterness suppressing flavone neohesperidosides. Certain hybrids, such as the K early, also contain bitter flavanone glycosides at levels which reduce its acceptability. Therefore, the knowledge of the relative type and

Table IV. Thin Layer Chromatographic Survey of Flavanones in Citrus Taxa

Citrus Taxa	Relative fluorescence intensity ^a									
	7-Rutinosides of:					7-Neohesperidosides of:				
	naringenin-4'-glucoside	isosakuranetin	eriodictyol	hesperetin	naringenin-4'-glucoside	naringenin	isosakuranetin	eriodictyol	hesperetin	naringenin-4'-glucoside
Citrus sinensis (7)	4	2	-	10	1	-	-	-	-	-
C. reticulata (4)	4	2	-	10	-	-	-	-	-	-
V. aurantifolia	-	-	-	10	-	-	-	-	-	-
C. limon "Lisbon"	2	-	10	10	-	-	-	-	-	-
"Meyer"	1	-	-	10	-	-	-	-	-	-
? "Ponderosa"	1	-	-	1	-	3	-	-	10	-
C. grandis	-	-	-	-	-	10	-	-	-	1
C. aurantium (3)	-	-	-	-	-	10	-	-	10	-
C. paradisi (2)	4	1	-	T	1	10	2	-	T	1
Poncirus trifoliata	-	-	-	-	-	10	10	-	-	-
Hybrids										
C. paradisi x C. reticulata										
"Minneola" Tangelo	4	2	-	10	-	-	-	-	-	-
"Orlando"	3	1	-	10	-	-	-	-	-	-
C. sinensis x Poncirus trifoliata										
"Rustic" citrange	4	4	-	1	-	5	10	-	-	8
"Troyer"	4	4	-	1	-	10	10	-	-	2

^a The flavanone with the highest fluorescence intensity was given a value of 10; T = trace; - = not detected.

() number of varieties tested

Source: (49)

concentrations of flavonoids in potential parentage will be important to plant breeders in developing hybrids of desired flavor.

Effects of Fruit Maturity, Rootstocks and Horticultural Variables

It is generally held that the amount of flavonoids in whole fruit increase only during the very early stages of fruit development. There is little change in the absolute amount of citrus flavonoids during most of the lifetime of the fruit. Kesterson and Hendrickson (7) found that the total amount of naringin (as measured by the Davis Test) in several cultivars of grapefruit remained constant once the fruit reached 2.3 inches in diameter. However, as shown in Figure 6, the concentration of naringin decreased continually because it was being diluted due to increasing fruit size. They (52) reported similar results with hesperidin in oranges. Huet (53) and Goren (54) conducted monthly measurements on the flavonoid contents of Valencia and Shamouti oranges grown in Morocco and Israel, respectively. They also found that the absolute whole fruit flavonoid content changed very little once the fruit reached a critical size early in its development.

While there is general agreement as to the whole fruit flavonoid content of citrus, there is considerable disagreement as to what, if any, changes occur in juice flavonoid concentrations as the fruit matures. Many investigators (7, 52, 53, 54) report finding no significant change in flavonoid content until after the fruit is past its peak maturity. These studies employed a variety of extraction techniques on most of the common orange and grapefruit cultivars that were grown in different regions of the world. Each used either the Davis test or the direct UV method to determine flavonoids.

An alternate view is that juice flavonoid concentration decreases continually during maturation. Lime et al. (55) using the Davis Test found flavonoid concentration in Texas Ruby Red grapefruit to decrease continually during the growing season. Hagen et al. (25) used a thin layer chromatographic-fluorometric method to measure the concentrations of each of the six major flavanone glycosides present in Texas Ruby Red grapefruit. They also measured total glycosides using the Davis Test. Since both they and Lime et al. (55) used the same variety of grapefruit grown at the same location, their results are compared in Figure 7. In both studies total glycosides decreased rapidly during the early part of the season and then gradually diminished as the fruit continued to mature. However, the differences in the Davis values, shown as curves A and D, for these studies are too great to be explained by seasonal variation. Comparing these values with another report (7) on the same cultivar and method of analysis it was found that the values of Hagen et al. (25) were unusually high. Since Hagen and co-workers separated the juice

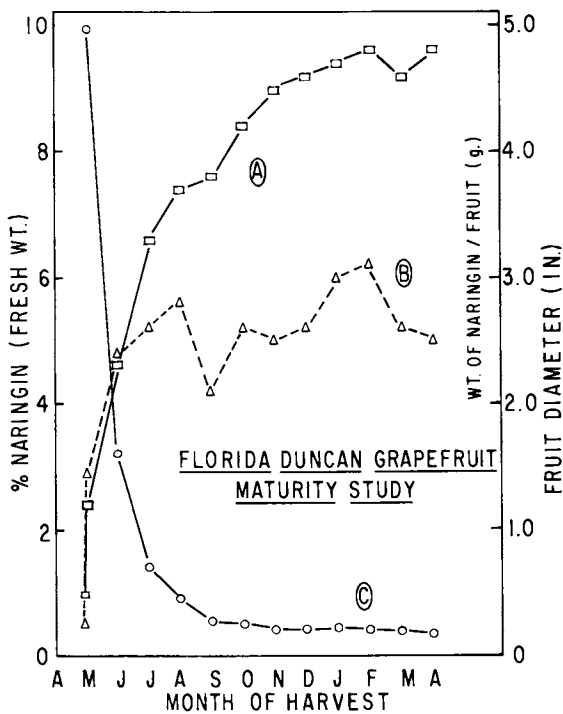


Figure 6. Effect of fruit maturity on: (A) fruit size; (B) weight of naringin per fruit; and (C) the % of naringin in each fruit (7)

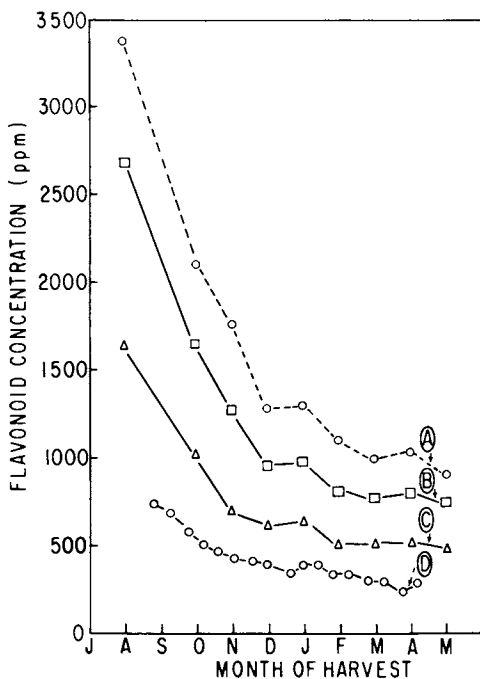


Figure 7. Maturity effects on naringin and other flavanone glycosides in juice sacs of Texas Ruby Red grapefruit; (A) "naringin" by Davis value; (B) total flavanone glycosides by TLC; (C) naringin by TLC (25); and (D) naringin by Davis value (55)

vesicles from the central core, segment membrane and albedo before extracting, there should be no contamination from these high flavonoid sources. Rouseff and Dougherty (56) found a small, but consistent, decrease of naringin in Florida grapefruit juice as the fruit matured. They also found that the major drop in flavonoid content as well as naringin, occurred after the fruit passed peak maturity. However, this was observed only when experimental conditions were tightly controlled, i.e., when a large number (120) of carefully sized fruit from the same bloom were extracted in exactly the same manner throughout the season.

Numberous factors, such as fruit size, horticultural conditions and the age of the tree (57) all affect the flavonoid content of the juice. The larger the fruit, the lower the flavonoid content. Rootstock effects are most evident early in the season (53) with only slight differences found at the end of the season. Horticultural practices such as spraying immature grapefruit with lead arsenate sprays for early maturity significantly increases the flavonoid content (58). The application of a growth regulator, such as gibberelin, to Shamouti oranges reduced their hesperidin content (59).

Climatic differences will alter the flavonoid content of citrus. Rouse et al. (60) and Maraulja et al. (61) found the hesperidin contents in Florida oranges to be higher in colder seasons. In a single season, Dougherty and Fisher (62) found the naringin concentration of grapefruit to increase dramatically after a freeze. Conversely, Herzog and Monselise (63) found the naringin content of grapefruit grown in warm arid regions to be lower than that from cooler, humid areas. Thus, cooler temperatures appear to favor increases in flavonoid concentrations.

Effects of Processing on Juice Bitterness

Flavonoids are not evenly distributed within citrus fruit. Flavanone glycosides are concentrated primarily in the albedo, (the inner, white, spongy portion of the peel) with lower concentrations in the central core and segment membranes. Juice contains the least amount of flavanone glycosides (7, 52, 53). Since these flavonoids readily dissolve in the juice, additional amounts of flavanone glycosides from other fruit parts may diffuse into the juice during extraction. In the production of orange juice, a higher flavonoid content is of little concern since most of this material will be tasteless hesperidin, the predominant flavonoid in sweet oranges. However, in grapefruit juice, the additional flavonoid content will generally produce a less desirable product because the principle flavonoid glycoside is the bitter naringin. Thus, the technological challenge in the production of good grapefruit juice is to minimize the amount of naringin incorporated into the juice from other fruit parts.

Extractor designs that minimize squeeze pressure and juice contact time with the albedo will produce grapefruit juices with

lower naringin levels. Designs which incorporate a prefinisher to separate segment membrane, central core and seeds from the pulp and juice immediately after extraction will also help to reduce juice naringin concentrations. It has been shown (52) that naringin concentrations could be reduced by limiting the amount of pulp incorporated into the juice. Juice naringin levels were reduced by as much as 20% when the pulp was immediately separated from the juice after extraction. The usual methods of pulp separation, however, produce juices with 10% less naringin but with no significant improvement in quality.

Extraction and finisher pressures can greatly influence the flavonoid content of citrus juices. Generally, as the fruit is squeezed harder more juice is recovered. However, excessive extractor pressures produce juice of a lower quality (35). As shown in Figure 8, grapefruit flavanone glycoside concentrations increase with increasing extractor pressures. In the early portion of the season almost twice as much naringin was obtained under hard squeeze conditions than was obtained with the soft squeeze. The effect of finisher pressure is not as clear, and is dependent on the composition of the raw juice which, in turn, is dependent on the type of extractor used.

Attaway et al. (64) found that extractor pressure was proportional to total flavanone glycoside values in several varieties of orange juice. In a later survey of commercial orange juice (65) it was found that there was a significant correlation between total flavanone glycosides (Davis Values) and flavor score. Since the flavanone glycosides in commercial orange juice are the tasteless rutinosides, the relationship observed is probably due to small amount of the bitter limonoid, limonin, whose concentration also increases with increasing extractor pressure.

At one time it was thought that methoxylated flavones were found only in the oil glands of the flavedo (2). However, later investigators (33) using more sensitive techniques found that they are distributed throughout the fruit. Rouseff and Ting (66) reported that of the five major methoxylated flavones in Valencia oranges, 96% were found in the flavedo and during extraction very little was incorporated into the juice from other fruit parts.

Thus, the type of extractor, extractor pressure, juice pulp contact time as well as ultimate juice pulp content will all alter the amount of flavanone glycosides found in the juice. It should be pointed out that if extraction conditions are carefully controlled, the quality of the juice will be maintained. No degree of manipulation of extraction conditions can yield a good quality juice if poor quality fruit is used. The important role that fruit variety and maturity play in determining the quality of the final product cannot be overemphasized.

Enzyme Debittering. One of the more promising ways to reduce naringin bitterness is to use enzymes to convert the bitter flavone neohesperidoside into a less bitter glucoside or non-bitter

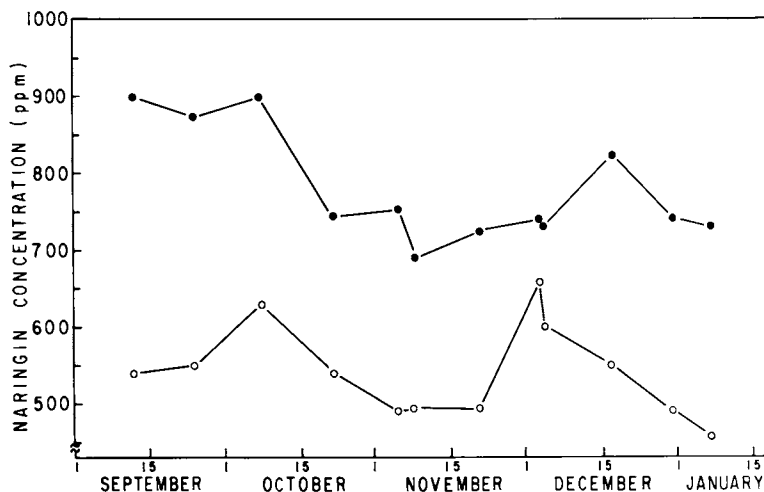


Figure 8. Effect of extractor pressure on total flavanone glycoside concentration for Florida grapefruit at different stages of maturity (35) ((●) hard squeeze; (○) soft squeeze)

Table V. Effect of Enzyme Treatment on Naringin Content and Bitterness Perception in Grapefruit Juice.(79)

Sample	Juice volume (mL)	Circulation time (min)	Naringin content (ug/mL)	Bitterness rating ^b
Control	--	--	285	3.8
1 ^a	1500	100-30	240	3.6
2 ^a	1500	180-134	195	3.3
3 ^a	500	120	122	3.2
4 ^a	500	125-150	95	2.9

a = blended samples of 2-6 batches of juice.

b = average bitterness scores from 20 member taste panel, where 1 = no bitterness, 2 = just perceptible, 3 = definitely perceptible, 4 = moderately intense, 5 = very intense and 6 = extremely intense.

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application of naringinase (73) and have done extensive studies to find inexpensive sources for this enzyme (74, 75). The most successful approaches have been to soak the peeled fruit in enzyme solutions for up to 16 hour before processing (76) or adding naringinase preparations with low pectinase activity along with the fruit as it is being canned (77). Roe and Brummer (78) successfully infused naringinase into whole shaved grapefruit (flavedo removed). This reduced the bitterness of the albedo to the point that it was as equally edible as the fruit segments. In addition, the process increased the nutritional and dietary fiber content of the final product.

Cost has not been the only problem in employing enzymes to reduce naringin bitterness. The other problem was the undesirable loss of cloud due to pectinase impurities in the naringinases employed (70). However, both problems have been reduced in a promising new procedure. Olson et al. (79) have successfully immobilized naringinase in a hollow fiber reactor to reduce naringin bitterness. Enzyme activity was not affected even after grapefruit juice was pumped through the hollow fiber reactor for up to three days. Thus, much more juice can be treated with the same amount of enzyme and the cost per unit of juice was reduced. Pectinase impurities did not cause a loss of juice cloud because the high molecular weight pectins could not diffuse through the hollow fiber membrane to come in contact with the enzyme. As illustrated in Table V, they could reduce juice naringin levels by controlling the amount and the circulation time of the juice. Naringin concentration was reduced from 285 to 95 ppm with a corresponding reduction in the bitterness rating. This is a promising approach to the problem of juice naringin bitterness, however, the process still remains to be evaluated under pilot plant conditions.

Enzymes have also been developed to reduce or eliminate problems associated with hesperidin's insolubility. Since hesperidin problems are visual problems, enzymes have been used to convert the insoluble hesperidin to the more soluble glycoside. The addition of these enzymes has greatly reduced the turbidity in canned mandarin sections due to hesperidin crystal formation during storage (80).

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The Role of Pectin in Citrus Quality and Nutrition

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Pectin is one of the major cellular structural components. It exists both in the primary cell wall and in the middle lamella, the intercellular cement between cells. In this capacity it contributes significantly to structural integrity of fruits and vegetables. As a soluble component of juice and an insoluble component of juice particulate material, pectin affects many facets of juice quality.

The basic structure of pectin consists of a chain of galacturonic acid units linked via $\alpha(1\rightarrow 4)$ glycosidic bonds. Although pure galacturonans have been reported (1), most pectin probably contains small quantities of rhamnose as inserts in the main chain (2). Soluble pectins may contain from 150 to 1500 units in the primary chain structure. In addition, most pectins have side chains containing arabinans, galactans, xylose and fucose (3, 4). These are attached by covalent bonding to the free hydroxyl groups of either the rhamnose inserts in the primary chain (5) or the galacturonic acid residues (6) (Figure 1). Recent advances in structural elucidation of pectin have been reviewed by Nelson (7).

In most pectins, many of the carboxyl groups of the galacturonic acid residues are esterified with methanol. The extent of esterification is expressed as the degree of esterification (0-100% D.E.) or methoxyl content [0-16.32%, the theoretical upper limit based on the molecular weight of a methylated galacturonic acid unit (8)]. Citrus pectins that are not deesterified by extractive procedures usually have a D.E. in excess of 50%. When combined, the factors of varying primary chain length, rhamnose inserts, side chains, and partial methylation yield a family of molecules of almost limitless heterogeneity. Any measurement made on a pectin solution is at best an average for the molecular species present.

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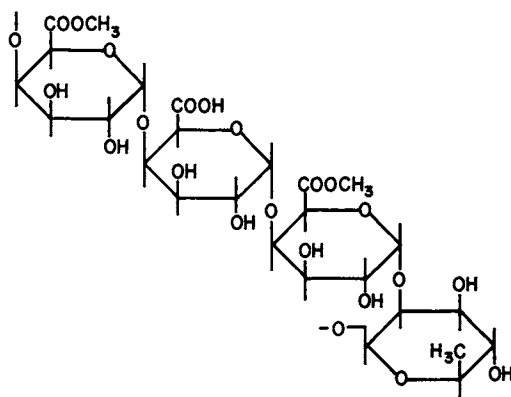


Figure 1. Segment of pectin molecule containing three galacturonic acid units and one rhamnose unit

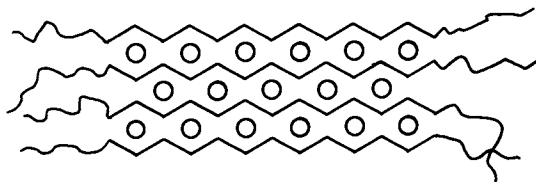


Figure 2. "Egg-box model" of calcium pectate formation. Calcium ions (represented by circles) bound by chain-stacked sections of deesterified pectin molecules. Adapted from Ref. 11.

The attributes of pectin which most directly affect citrus product quality--viscosity, gelling ability, and ability to precipitate as pectates--are primarily determined by chain length (molecular weight) and degree of esterification. High molecular weight, high D.E. pectins form gels when soluble solids levels reach 65% and pH is in the range of 2.9-3.4 (9). Low D.E. pectins will gel without sugar and at high pH if sufficient calcium is present. If molecular weight is lowered below a certain level, pectins lose their ability to gel in either system. Precipitation of pectins as pectates and gel formation are influenced not only by D.E. and molecular weight, but also by the distribution of free acid and esterified sites along the molecule (10). Rees (11) and Grant et al. (12) proposed an "egg-box model" to explain calcium pectate formation (Figure 2). In this model deesterified sections of pectin molecules chain stack, cooperatively binding calcium ions in the intervening cavities.

Pectin is often divided into three categories on the basis of solubility (13). Water soluble pectin, that which can be extracted from tissue with hot water, usually has a high D.E. (2). Insoluble pectates are only soluble if chelating agents such as ammonium oxalate or detergents are present. Insoluble pectates are low D.E. pectins which have formed the insoluble salt of a divalent cation, such as calcium. The third category, protopectins, are solubilized only by heating with acid or alkali. The chemistry of this class of pectins is poorly understood, since harsh extraction procedures alter both molecular weight and D.E. The insolubility of protopectins *in situ* is probably a result of bonding to hemicelluloses and mechanical intermeshing with other cell wall constituents (14).

Citrus fruits, especially certain of their component parts, constitute one of the richest sources of pectin. On a dry weight basis, as much as 30% of orange fruit albedo may be pectin (8). The rag, comprising the fruit core and segment membranes after juice extraction, is also a rich source. Since pectin is a cell wall component, it follows that comparatively little would be present in juice expressed from fruit. For example, concentrations ranging from 0.01 to 0.13% in orange juice have been reported (15). Much of this would be present as cell wall fragments and particulate material in juice pulp and cloud. Even at these levels, pectin influences juice quality, both positively and negatively, by its contribution to viscosity, gelation, and cloud stability.

Quality

Viscosity. In freshly extracted juice, viscosity imparted by pectin is a desirable characteristic, commonly referred to as body. If this viscosity is not present, or is lost through destruction of the pectin colloid, juice is described as thin,

watery, and lacking a juice-like mouth feel. Fresh, single-strength, or reconstituted juices should, therefore, contain some soluble pectin for optimum quality.

However, when juice is processed into concentrate, excessive pectin-induced viscosity can be detrimental. Increasing energy costs for frozen storage and transport of concentrate make production of very high Brix concentrates economically attractive. Concomitant high viscosity is a technological problem which must be dealt with if these products are to be accepted. As total soluble solids approaches 65%, viscosity increases rapidly. Ingram (16) noted that this level coincides with the sugar level required for sugar-acid-pectin gels. Such viscous preparations cannot be poured or reconstituted to single-strength juice easily. To obtain flowable high solids concentrates, soluble pectin levels must be reduced either before or during concentration. This may be accomplished by treating juice before concentration with a commercially available polygalacturonase (PG) preparation (17). PG hydrolytically cleaves primary pectin chains between adjacent nonesterified galacturonic acid residues, reducing viscosity rapidly at 30°. Subsequent pasteurization terminates added PG and native pectinesterase (PE) activities, stabilizing juice cloud.

An alternative approach is to treat concentrate to reduce viscosity. Berk (18), using ultrasonic radiation, succeeded in lowering viscosity of 60° Brix concentrate to 25% of its initial level. Viscosity of 70° Brix concentrate prepared from this material was only 50% higher than that of untreated 60° Brix concentrate. Viscosity of 70° Brix concentrate prepared without ultrasonic treatment was nearly 8 times that of 60° Brix concentrate. Irradiation was more effective at an intermediate stage of concentration (e.g., 60° Brix) than after concentration to 70° Brix.

Viscosity poses a similar problem in the production of pulp wash concentrate. Pulp wash consists of juice solids obtained by countercurrent washing of pulp after its separation from juice. On a °Brix basis, pulp wash liquids are higher in pectin than juice from which the pulp has been screened (19). Concentration of pulp wash above 40° Brix is at times hampered by excessive pectin levels (20). To control viscosity processors may be forced to reduce finisher pressure to minimize pectin extraction, thereby curtailing yield. A more effective solution is to treat pulp wash with pectinases to reduce pectin levels (21). If pectinases are incorporated into the wash water, this method has the advantage of increasing total solids yield by reducing juice retention in the pulp.

Gelation. Pectins can form two types of gels: 1) sugar-acid-pectin gels, with a 65% sugar level, low pH, and highly esterified pectin, and 2) pectate gels, requiring only low

ester pectin and divalent cations. The former would be seen only in very high Brix concentrates. Gelation seen in commercial concentrate (42-45° Brix) is of the latter type, and varies from soft curds to firm gels. This is a visual quality defect, but in severe cases firm gels impair reconstitution of concentrate. Gelation is accompanied or preceded by clarification, since both phenomena involve precipitation of low D.E. pectins with calcium.

Gelation became a significant problem with the introduction of frozen orange juice concentrate, which initially was often packed without pasteurization. This product, when not frozen, rapidly developed low ester pectins by virtue of the PE it contained. Much of the research generated in response to this problem has been reviewed by Joslyn and Pilnik (22). Briefly, it has been found that gelation is the result of pectin deesterification (23), and increases as juice pulp (24), PE (25), and extraction pressures (24) increase. In concentrate undergoing gelation, water soluble pectins tend to decrease, while pectates increase (22). Gel formation is best prevented by pasteurization at both sufficient temperature and holding time to inactivate most PE, coupled with storage of concentrate near -18°. Introduction of high temperature evaporative systems has largely eliminated the problem of gelation.

Gel formation by pectins can be a positive quality factor in some citrus products, for example, gelled citrus salads (26). These salads consist of citrus fruit sections suspended in a gel of low ester pectin, carrageenan, and locust bean gum. Similar pectin mixtures have also been suggested as a sealer for grapefruit halves (27). Ready-to-eat grapefruit halves may be satisfactorily stored 48 hours if chilled. Prolonged storage results in flavor deterioration, drying and fungal growth. A stiff citrus flavored pectin gel applied to the cut surface sealed the fruit, maintaining flavor and preventing shrinkage or fungal invasion for up to 19 days. This would be of particular value to institutional consumers.

Juice Cloud. Mechanical extraction of citrus fruits yields a turbid suspension of wall fragments and cellular organelles in a serum composed primarily of cell vacuolar fluids. In most citrus juice products, such a suspension of fragments and organelles is a desirable component, since it provides most of the characteristic color and flavor (28). Essence and peel oils suspended in juice contribute desirable citrus notes to flavor, and these oils are rapidly adsorbed by juice particulate material shortly after extraction (29).

If steps are not taken to stabilize cloud, most citrus juices will clarify when allowed to stand. Clarification occurs when native PE lowers the ester content of juice soluble pectin until it becomes susceptible to precipitation as insoluble pectates (23). If pectin levels are high enough, as in concentrates, these pectates may form a gel.

At lower levels, they settle out as a floc, occluding cloud particulates and removing them from suspension (28). Estimates of the D.E. necessary for such precipitation have been made, ranging from 38% for a 25% cloud loss (23) to 27% in juice which was 85% clarified (30). However, the critical ester level at which pectin precipitates as pectate cannot be established from assays on the D.E. of total juice pectin. Pectins exhibit a natural heterogeneity with respect to ester content (Figure 3a), and when this is coupled with nonrandom blockwise removal of methoxyl groups by PE (10), a wide range of ester contents may be expected (Figure 3b). A closer approximation of the critical D.E. associated with clarification can be made by examining pectins fractionated according to ester content. In such a study, Baker (31) found that pectin fractions with D.E. of 14% or less were able to clarify juice, whereas those with D.E. of 21% or more were not. This indicates that the critical D.E. for precipitation of pectins as pectates lies between 14 and 21%.

Many early researchers of citrus juice cloud presumed that a soluble pectin matrix supported and stabilized cloud; destruction of this matrix was therefore seen as a contributing cause of clarification (32). More recent work involving aqueous suspensions of cloud showed pectin is not necessary for cloud stability (33, 34). Orange juice cloud is quite stable suspended in water, and can only be made to clarify if pectin, calcium, and sufficient KCl to solubilize particulate-bound PE are added (33). Even though pectin is not necessary for cloud support, soluble high ester pectin may delay clarification by "substrate flooding", that is, by diluting PE activity. Rouse et al. (35), studying frozen orange juice concentrate, found that packs with high pectin level were more cloud stable than packs with low pectin level at corresponding PE levels. Pectin may also retard clarification by inhibiting coacervation of cloud particulates and precipitating pectates (36).

Although the addition of pectin may retard clarification, this action does not represent a practical approach to stabilizing cloud. To achieve cloud stability, deesterification of soluble pectin to low ester pectin and precipitation of the latter as pectates must be prevented (Figure 4). This sequence may be disrupted by enzymic destruction of either pectin or low ester pectin, by blocking the precipitation of pectates, or by inactivation of PE. Commercially, inactivation of PE by heat is the cloud stabilization procedure of choice, since heat is also needed for pasteurization. In acid juices, spoilage organisms are more easily inactivated than is PE, so cloud stabilization requires higher temperatures than pasteurization (37). Thermal inactivation of PE is not linearly related to stability. Guyer et al. (38) found most PE in orange juice could be destroyed without an appreciable effect on cloud stability; a further small decrease in PE

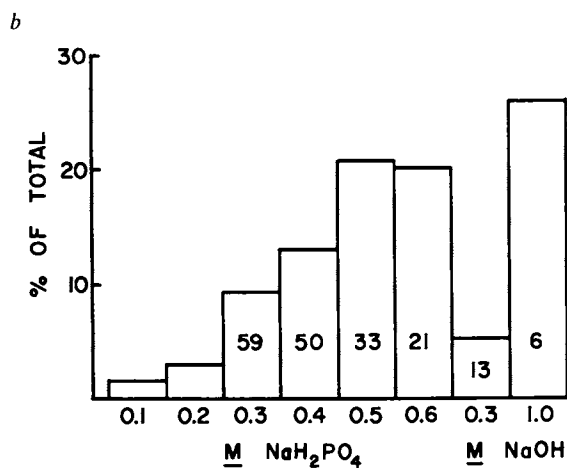
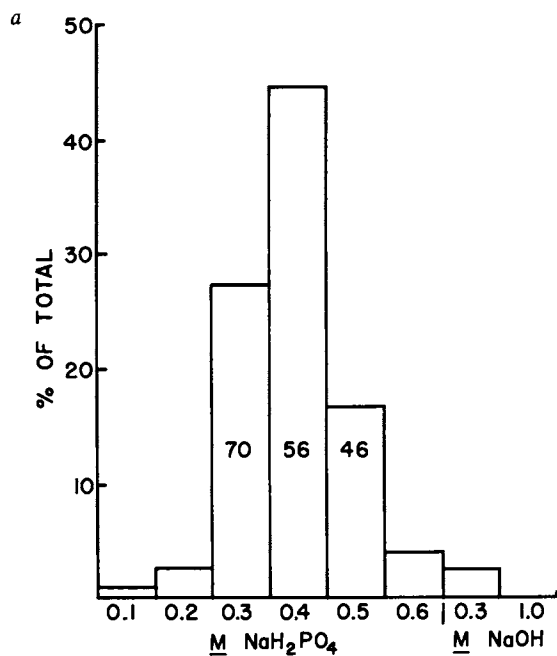


Figure 3. Degrees of esterification of fractions from (a) citrus pectin separated on DEAE-cellulose (adapted from Ref. 6) and (b) PE-treated citrus pectin separated on DEAE-cellulose

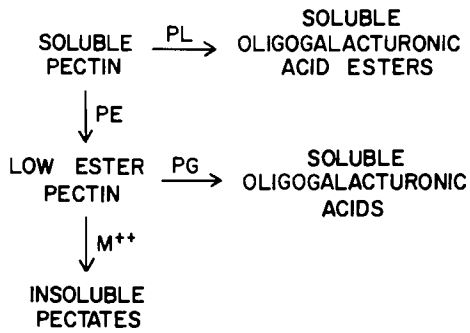


Figure 4. Possible pathways of pectin degradation in juice

activity resulted in substantial cloud stability. This nonlinear response was explained when Versteeg (39) found that orange juice contains multiple forms of PE. One of these, a heat stable high-molecular-weight isozyme, accounted for about 10% of total PE activity. Presumably it is this form which contributes to cloud loss in incompletely stabilized juice.

Enzymic depolymerization of juice soluble pectin before it can be deesterified by PE also stabilizes cloud (30). This has been accomplished with pectin lyase (PL), an enzyme which degrades esterified pectin to oligogalacturonic acid esters. Since PL cannot attack deesterified pectin, cloud stabilization of PE active juice requires relatively large quantities of PL to compete with PE for esterified pectin.

Cloud loss may also be prevented by enzymic destruction of low ester pectins prior to their precipitation as pectates. Commercial fungal PG preparations (40, 41) or PG derived from yeast (42) have been used successfully to stabilize cloud of unpasteurized PE active juice. As only the low ester pectins need be destroyed to prevent cloud loss, considerably less PG is required than PL (30). Cloud stabilization with pectinases permits production of cloud stable juices with lower pasteurization temperatures (40).

For low ester pectin to precipitate as pectate and clarify juice, divalent cations such as Ca^{++} must be available to participate in the coacervation (36). The use of hexameta-phosphates to sequester Ca^{++} ions has been suggested as a means to block pectate precipitation and stabilize cloud (43).

Clarified Juice. Some citrus juices, notably lime and lemon, are in demand as clarified products. Natural clarification, in combination with filtration, is often used to achieve a sparkling clear serum. However, native PE action is slowed by the high acidity of these juices, and may not give satisfactory cloud removal. In this instance, modified pectins can serve to enhance juice quality by removing cloud. Low ester pectins obtained by treatment of citrus pectin with either PE or NaOH clarify citrus juices over a wide pH range (44). Ester content for optimum clarification depends both upon juice pH and method of deesterification. Conversion of all esterified carboxyl groups in the pectin chain to free acids yields polygalacturonic acid, which is largely water insoluble. If this acidic polymer is brought into solution by partial neutralization with base, it can also function as a clarifying agent for citrus juices (45). Cloud reduction with polygalacturonic acid preparations is also influenced by juice pH, but in this instance polymer molecular weight determines pH optimum.

Intact Fruit and Sections. A number of studies have been made of pectic substances in citrus fruit during growth and maturation. These have been reviewed by Kefford and Chandler (46) and by Rouse (15). In general, once fruit has reached full size, few notable changes are seen during maturation in the balance of water-, oxalate-, or NaOH- soluble fractions of pectin, or in its ester content. This may in part be due to the fact that citrus fruit lacks a true climacteric ripening with its attendant physiological changes. Freeze damage to fruit affects its pectin content and distribution among water-, oxalate-, and NaOH-soluble fractions, but the extent of the changes depends largely on fruit variety (15). Valencia oranges, for example, apparently are able to partially recover in the interim between winter freeze and late Spring harvest.

Canned citrus sections consist of excised fruit segments, normally with segment membrane removed, packed in water or syrup. Firmness and freedom from broken sections are primary determinants of quality in this product. Section firmness is dependent upon their content of oxalate- and NaOH-soluble pectin (15). Low ester pectin added to grapefruit sections in water did not improve firmness or reduce section breakage, but did improve drained weight (47). However, calcium salts (as calcium lactate) added to syrup covering grapefruit sections significantly increased the percentage of firm sections (48). This would be analogous to the well known calcium firming of canned tomatoes.

Nutrition

Interest in pectin from a nutritional standpoint has increased with new evidence of its influence on several physiological processes. Long acknowledged as an effective antidote to diarrhea, pectin has now been found to be of possible benefit in control of cholesterol levels and in management of diabetes. As one of the richest potential sources of pectin, citrus fruits could enjoy enhanced nutritional status from these findings.

Metabolism of pectin. Pectin has only recently come to be considered a part of the dietary fiber complex. Previously it was excluded because 1) it is not fibrous (except at the molecular level), 2) it escapes detection in standard fiber tests owing to its solubility, and 3) it usually does not survive intestinal passage. In a reassessment of which dietary components should be considered fiber, Trowell (49) proposed that dietary fiber include those constituents of food resistant to hydrolysis by man's alimentary enzymes. Spiller (50, 51) suggested that confusion surrounding the term "fiber" be avoided by using the term "plantix" to denote those plant materials of polymeric nature not attacked by human digestive enzymes.

Regardless of terminology, pectin qualifies as fiber under these definitions. Kertesz (52) showed that human saliva, gastric juices, trypsin, and pepsin were ineffective in hydrolyzing pectin. Werch and Ivy (53) demonstrated that pectin passes relatively unchanged through the stomach and ileum. Yet little if any pectin can be recovered in the stool, due to rapid digestion by bacterial enzymes present in the colon. Breakdown products include galacturonic acid, volatile acids such as formic and acetic, and finally, carbon dioxide and water. Although galacturonic acid is not absorbed by the human ileum or colon, it does not appear in the feces (54). Apparently this acid is further degraded to acetic and formic acids, which have been seen to increase in fecal excretion of subjects fed 30 g of pectin daily.

Despite the rather complete digestion of pectin in the colon, there is some question as to the energy derived therefrom. Viola et al. (55), studying weanling rats, reported pectins' contribution to digestible energy was negative. This was supported by Hove and King (56) who used a rat growth bioassay to demonstrate pectin provided no measurable energy to young rats. In contrast, Campbell and Palmer (57) cite two studies (one on rats, the other on humans) which showed a net digestible energy for pectin. To the extent that volatile acids resulting from colonic pectin degradation are absorbed, pectin would contribute to caloric intake. Whether this results in a net energy gain depends on other pectin-induced effects. In rats, increasing dietary pectin levels decrease digestive efficiency, protein absorption (56), and lipid absorption (55). Positive or negative digestible energy values probably depend on the degree to which pectin interferes with absorption of other dietary components.

Antidiarrheal Effect. Use of pectin (in the form of scraped apples) has been acknowledged as a remedy for diarrhea for over 200 years. Its efficacy is attested to by commercial antidiarrheal preparations based on pectin. Despite this long history of use, very little is known of the mechanism by which pectin promotes a return to normal bowel function. It has been suggested that pectin counteracts diarrhea by absorption and removal of toxins, or by enlargement of stool volume and sweeping of the colon with a formed stool (9). In a stable digestive system pectin could not absorb and remove toxins, as it is largely degraded in transit. Pectin is also much less effective than other fibers in increasing stool bulk: even at 36 g/day intake, Cummings et al. (58) found stool weight increased only 33%. However, during intestinal upset, normal colonic retention times are greatly reduced. As a result, pectin is not digested and can be recovered in the stool (9). Therefore it could conceivably function to some extent in the manner described above.

Alternatively, Werch and Ivy (54) suggest the favorable effect of pectin may be due to inhibition of undesirable organisms by volatile acids (formic and acetic) derived from pectin breakdown. Such acids promote a return to normal bowel pH, and are bacteriostatic against many organisms (57). Werch and Ivy also hypothesized that offending organisms may be starved out or overgrown by desirable organisms which use pectin as a source of food. Much more work is needed to establish the mechanism governing the antidiarrheal effect of pectin.

Hypocholesterolemic Effect. No effect of pectin has received more attention in recent years than its ability to reduce serum cholesterol levels. Altogether there have been a dozen reports relating dietary supplementation with pectin to serum cholesterol levels in human subjects (see 59). The first of these reported a study of middle-aged men whose normal diets were augmented with a daily ration of biscuits containing 15 g of either cellulose or pectin (60). Cellulose did not lower serum cholesterol; citrus pectin did, to a level about 5% below control levels. Pectin has also been administered as pills (61), in water (62), or as a gel with added fruit (63). The cholesterol-lowering effect of pectin was greatest when administered as a gel (59). Levels have varied from 2 g/day (61) to 40-50 g/day (64). Palmer and Dixon (61) reported serum cholesterol levels of human subjects with normal cholesterol levels were significantly lowered by pectin intake of 6-10 g/day, but not 2-4 g/day. Pectin is particularly efficacious in lowering serum cholesterol levels of hypercholesterolemic patients (64). Delbarre et al. (65) were unable to reduce serum cholesterol levels significantly with 6 g/day, and concluded pectin did not lower serum cholesterol. However, some reduction was observed. The 6 g/day level used by these authors was the lowest level providing significant cholesterol reduction observed by Palmer and Dixon. The conclusions of Delbarre et al. (65) have been refuted by Kay et al. (66).

In studying results from both chickens and man, Fisher et al. (67) concluded that pectin has a hypocholesterolemic effect only when fed with dietary cholesterol. On cholesterol-free diets, plasma cholesterol is not affected by dietary pectin. Subjects fed pectin with a cholesterol-containing diet had plasma cholesterol levels that were lower relative to those of subjects on the cholesterol control diet, but not relative to those of subjects on a cholesterol-free control diet.

There are several ways pectin could reduce serum cholesterol. In studies with human subjects, fecal excretion of bile acids, fatty acids, and total steroids increased when subjects were fed 15-40 g/day of pectin (58, 63, 64). Since pectin usually lowers serum cholesterol only when cholesterol is present in the diet, it seems that pectin might act by reducing cholesterol absorption. Several groups have found that in rats dietary

pectin does indeed reduce cholesterol absorption. Using cholesterol-4-¹⁴C, Leveille and Sauberlich (68) and Kodama et al. (69) showed cholesterol absorption was impaired by dietary pectin. Kelley and Tsai (70), who also reported a decreased cholesterol absorption in pectin fed rats, concluded that pectin acted by interfering with cholesterol absorption and by increasing cholesterol turnover. However, Leveille and Sauberlich found rat liver and serum cholesterol levels were lowered even when pectin and cholesterol were fed on alternate days. From this the authors concluded that although pectin did impair cholesterol absorption, it primarily affected cholesterol levels by inhibiting bile acid absorption. This hypothesis appears to be favored (71, 72).

Bile acid reabsorption may be impaired by binding with pectin during transit of the ileum. Selvendran (73) observed a greater adsorption of sodium cholate by vegetable fibers containing pectin than by fibers containing lignin. Nagyvary and Bradbury (74) have proposed a model for bile salt binding by pectins or alginates complexed with Al⁺⁺⁺ ions. In support of their hypothesis, the authors demonstrated strong hypocholesterolemic activity for a pectin-aluminum complex. Furda (75) has tested and extended this hypothesis, showing that Fe⁺⁺⁺ complexes of natural insoluble pectinaceous fiber (citrus albedo) are even more effective than Al⁺⁺⁺ complexes in binding fatty acids. Using an equilibrium dialysis technique, Baig and Cerda (76) were unable to demonstrate binding of bile salts to soluble citrus pectin. This agrees with the findings of Furda (75), who also found no binding of fatty acids to insoluble pectin complexed with H⁺, Ca⁺⁺, or Mg⁺⁺. The effectiveness of pectin in binding bile salts may well depend on the extent of its complexing with trivalent cations.

Impairment of bile acid absorption and consequent loss of these acids via excretion presumably causes an increase in hepatic conversion of cholesterol to bile acids. This conversion lowers serum cholesterol, particularly when serum contains high levels of cholesterol derived from dietary intake. However, when fed with a cholesterol-free diet, 10% pectin supplementation stimulated a 3-fold increase in cholesterol biosynthesis (77). Biosynthesis of phospholipids and triglycerides also increased significantly; hence, it was suggested that these increases occurred in response to diminished fat absorption occasioned by pectin intake. This compensatory biosynthesis of cholesterol and lipids may account for pectin's inability (in most cases) to lower serum cholesterol levels in animals fed cholesterol-free diets.

Regrettably, most studies relating dietary pectin to hypocholesterolemia include no data on molecular weight or D.E. of the pectin consumed. These omissions occur despite a report as early as 1962 that high-molecular-weight, high D.E. pectins were more effective in reducing rat liver cholesterol levels

(78). Pectin with 62% D.E. largely counteracted the increase in liver cholesterol levels brought about by dietary cholesterol. Low ester pectin (30% D.E.), polygalacturonic acid (PGA), and 50% esterified PGA were all without effect. Conflicting reports on the efficacy of pectin as a hypocholesterolemic agent may be due to variations in these parameters. For example, citrus pectin was found to be more effective than tomato pectin in lowering serum and hepatic cholesterol levels of rats (79). D.E. values for the citrus and tomato pectins were 56 and 40%, respectively. Thus, differences in D.E. levels rather than pectin source could have accounted for the superior effectiveness of citrus pectin. Mokady (80) and Judd et al. (81) confirmed that serum cholesterol reduction is most pronounced with high D.E., high-molecular-weight pectin (Table I).

Table I. Effect of Pectin on Rats Fed a Cholesterol-Free Diet (54).

	Relative values (% of control)		
	Blood cholesterol	Fecal lipids	Fecal sterols
Control (no pectin)	100	100	100
Low MW pectin	91	458	278
Low ester pectin	86	390	222
High MW pectin	76	735	372

Recently the means by which pectin lowers cholesterol levels and even the validity of this effect have been questioned. Upon finding no bile salt binding capacity for soluble pectin, Baig and Cerda (76) proposed that pectin lowered serum cholesterol levels by forming insoluble complexes with the serum low density lipoproteins (LDL) which transport circulating cholesterol. Complexing of LDL by citrus pectin was observed *in vitro*, but the way in which pectin or some component thereof enters the blood stream to effect such binding *in vivo* has not been determined.

Pfeffer et al. (82) have found that bile salt binding activity of commercial citrus pectins was lost if these products were dissolved, filtered, centrifuged, and reprecipitated before testing. Binding activity was concentrated in the residue pellet from centrifugation, which was found to be fine diatomaceous earth. This contaminant was probably introduced during filtration steps in processing and purification of pectin. The authors concluded that any hypocholesterolemic effect of commercial pectin was due solely to its diatomaceous earth contamination.

Glucose Tolerance. Ingestion of carbohydrates temporarily increases serum glucose levels and, in response, serum insulin levels. Diminished insulin levels of diabetics permit an excessive blood glucose rise, with urinary excretion of glucose. In 1977 Jenkins et al. (83) reported that both hyperglycemia and insulin peaking after consumption of carbohydrates were reduced by dietary pectin. Addition of 10 g of pectin to food reduced blood glucose measured 15 min after eating, and decreased the insulin peak for 30 min. Iwasaki et al. (84) also reported on the possible use of pectin in treatment of diabetes. These authors observed a significant reduction of urinary glucose excretion when 15 g/day of pectin was incorporated in the diet. Consumption of 8 g of pectin with 30 g of glucose in peeled oranges lowered the blood glucose response when compared to consumption of an energetically equivalent quantity of orange juice containing only 0.2 g of pectin (85). Similar results with apples, apple puree, and juice have been discussed by Heaton (86). Recently Holt et al. (87) reported that normal subjects fed 50 g of glucose with 14.5 g of pectin had lower blood glucose levels than controls. The authors hypothesized that the depression of glucose response was an indication of delayed absorption. To test this hypothesis, they fed subjects pectin with acetaminophen. Absorption of acetaminophen was also slowed by pectin, but total absorption was unaffected. Further, depression of the blood glucose response by pectin did not occur in a patient whose stomach had been removed. Thus, it was suggested that pectin may moderate the glucose (and hence the insulin) response by delaying the rate at which stomach emptying occurs.

Detoxication of Metals. Pectins or pectin derivatives have been proposed as antidotes for heavy metal poisoning for nearly 200 years. Kertesz (9) has reviewed the early work in this area. Pectin complexes lead so strongly as to quantitatively remove it from solution. Absorption of lead, arsenic, and selenium by several animal species has been reduced by pectin- or apple-containing diets. Use of pectin as a prophylactic agent in lead poisoning continues to be of interest. Bondarev (88) recently reported an increase in excretion and a decrease in bone accumulation of lead when rats fed 6 mg/day of lead also received 72-432 mg/day of low ester pectin. Paskins-Hurlburt et al. (89) achieved an 87% decrease in lead absorption by pectate fed rats. These studies bear out the early observations of Fellenburg (see 9, p. 572), who concluded that pectin of decreased ester content would have an enhanced ability to complex metals.

In concluding this review of potential nutritional applications of pectin, one might ask: Can levels of pectin comparable to those used in these studies be obtained by a reasonable consumption of fruit or fruit products? The answer

would appear to be a qualified yes. Truswell, for example, cites two studies attributing a hypocholesterolemic effect to the daily consumption of several apples (59). Kay and Stitt (85) slightly reduced the hyperglycemic response to glucose with whole oranges. However, subjects consumed 624 g of fruit, and fruit was "thinly peeled", presumably leaving much of the albedo for consumption.

In most citrus fruit products as currently consumed, pectin content would not meet even the minimum levels found effective in nutritional investigations. Those portions of citrus richest in pectin--the peel, core and segment membranes--are usually discarded as inedible. Juice pectin levels are far too low to allow for the necessary intake. Juice sacs or pulp of grapefruit as normally eaten contain only 0.3% pectin on a fresh weight basis (90). An intake of 6 g of pectin [the minimal dose giving a significant hypocholesterolemic effect (61)] would require consumption of 2 kg/day of grapefruit. By comparison, grapefruit peel is approximately 3.5% pectin on a fresh weight basis (91). Since 40% of a grapefruit may be peel, a 500 g fruit would contain 7 g of pectin in the peel alone. Edibility of this peel is diminished in part by presence of the bitter compound naringin in the albedo, and in part by oil from glands in the flavedo. These obstacles to consumption of peel have been partially overcome with a procedure described by Roe and Bruemmer (92, 93). Flavedo of fruit is removed with a mechanical peeler, and albedo is vacuum infused with a solution containing naringinase, sugar, flavor, color and gelatin. Naringinase reduces bitterness; sugar, flavor, and color enhance palatability and acceptability; and gelatin reduces leakage of the infused solution. This treatment improves the hedonic rating of grapefruit peel from inedible to marginally acceptable (93). With further refinement, this technique could perhaps make available a new dietary source of pectin. Nutritional benefit could thus be obtained from a source now considered as waste.

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Color as Related to Quality in Citrus

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Peel color is probably the most important factor in determining whether consumers purchase fresh citrus fruits. Normally, the purchaser does not taste the fruit before buying, and has come to associate a bright peel color with a highly palatable interior. Although this conclusion may not always hold, everyone from the producer to the retailer tries to uphold this belief. The color of citrus processed products is also important. Advertisers have been very successful in relating sunshine and bright orange color of orange juice with good health. Hence, highly colored juice brings a premium price. With the importance of color in citrus established, it is the purpose of this review to describe some of the factors that contribute to color, how color can be controlled, relationship between color and vitamin A and grading for color.

Chlorophyll in Peel

The color of citrus peel is due mainly to chlorophyll and carotenoids. Chlorophyll is responsible for the deep green color considered typical of premium quality limes (1). For all other types of citrus, chlorophyll is considered an unsightly and unwanted contaminant. For many years, an effort has been made to rid ripe fruit of chlorophyll. From the time fruit sets on the trees at blossoming until it commences to mature, the peel is green. Following the first cool nights in the fall there is a color break in the peel of early-maturing cultivars. Some factors known to be associated with delay in fruit coloring at time of maturity are: use of high rates of potassium (2) or nitrogen (3) in the fertilizer, spraying with gibberellin (4), or benzyladenine (5).

The most important factor related to chlorophyll breakdown in citrus peel is cool temperatures. According to Stearns and Young (6), a color break results from temperatures below 13°C in Florida. In California studies, the daytime air, the nighttime air and soil temperatures were all found to be important

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for peel color (7). With temperatures of 20°C for daytime air, 7°C for nighttime air and 12°C for soil, brightly colored fruit were produced. Higher temperatures for any one of these conditions resulted in less orange color for Valencia oranges. Sonnen *et al.* (8) observed the effect of root temperatures on the fruit color of two citrus cultivars. When comparisons were made with satsumas at 14°C and 30°C and with calamondins at 12°C and 25°C, there were higher amounts of carotenoids and better color in the peel from fruit grown at the lower root temperatures.

It is not uncommon for fruit grown in the tropics to have a green peel even when mature. Green or mostly green is the accepted color of citrus fruit grown at low latitudes and sold in markets around the world. The reason for the green peel in the tropics is that high temperatures prevent the breakdown of chlorophyll. In controlled postharvest experiments, Wheaton and Stewart (9) found when citrus fruit was stored at 30°C there was no loss of green color; at 25°C there was some loss; at 20°C there was a decided color break and at 15°C most of the chlorophyll disappeared in 4 weeks. These studies were performed with less than 0.005 ppm ethylene produced by the fruit. Vines *et al.* (10) also reported only traces of ethylene were found in the internal atmosphere of unstressed citrus fruits. Other studies have indicated citrus fruit give off 0.007 to 0.110 $\mu\text{l}/\text{kg}/\text{hr}$ of ethylene (11). The low levels of ethylene would suggest only threshold amounts exist in fruit and the activity of these ethylene concentrations is highly temperature sensitive. At higher temperatures, i.e. 30°C, the endogenous concentrations of ethylene are not sufficient to bring about chlorophyll breakdown. However, when excess ethylene (10 ppm) is supplied to green fruit, then chlorophyll breakdown takes place at a faster rate at 30°C than at lower temperatures. A partial answer to this phenomenon may be the differential degradation of chlorophyll-a and chlorophyll-b. Shimokawa (12) discovered there was preferential breakdown of chlorophyll-b when satsuma mandarins were degreened with ethylene. Conversely, chlorophyll-a was more predominantly degraded than chlorophyll-b in the non-treated fruits. Similarly, Jahn and Young (13) observed that citrus fruit with a deep green color could not be satisfactorily degreened with ethylene. They assumed this was due to a high residual chlorophyll-b level. They pointed out that tangerines, which are easily degreened, have an a/b ratio near 3, whereas, Hamlin oranges, which are more difficult to degreen, have a ratio near 2.

Degreening of peel has been related to chlorophyllase activity. Barmore (14) demonstrated that chlorophyllase activity in peel increased following treatment of fruit with ethylene. This increase in enzyme activity was associated with chlorophyll degradation. These observations have been confirmed by Japanese studies (15).

Shimokawa *et al.* (16) examined the changes in chloroplast structure induced by ethylene in satsuma mandarin. Electron micrographs showed peel of ethylene-treated fruit had fewer chloroplasts and of smaller size. The inner membrane system of the chloroplasts was found to disintegrate prior to the breakdown of other cell structures.

Commercial Aspects of Degreening

It has been a common practice for almost a hundred years to treat green splotchy fruit in various ways to overcome the green color. At first, it was customary to "cure" the fruit with kerosene heaters (17). However, in 1923 Denny (18) recognized that ethylene was the constituent in the burned kerosene that caused degreening. Degreening with ethylene is a common practice in most places where citrus is grown. It is generally carried out by taking the fruit directly from the orchards in ventilated boxes and storing in large rooms. In Florida, the temperature is maintained between 29°C and 30°C and the relative humidity at 90 to 96%. Ethylene is supplied at a rate of 1 to 5 ppm and air is circulated throughout the rooms with at least one change per hour (19, 20). Degreening is generally carried on for 36 hr or less. Similar conditions are used in most places except the ethylene concentration and temperature may vary.

Cohen (21) has suggested the use of an "intermittent" exposure to ethylene and heat for degreening. The method consists of treating fruit with 10 ppm of ethylene at 25°C for 12 hr intervals. During the 12 hr interruption, the ethylene dropped to 0 to 1 ppm and the temperature dropped 1 to 2°C. Using this procedure, there was a decrease in respiration rate with less loss in fruit weight. There was less decay and the color development was similar to fruit treated by continuous degreening.

In Japan, degreening of satsuma mandarins is a relatively recent practice, becoming important in about 1970. According to Kitagawa *et al.* (22), two methods are used: (a) the common trickle system used in large packinghouses where the fruit is exposed to 5 to 10 ppm ethylene; (b) the system used by individual growers consists of treating fruit with 500 to 1000 ppm ethylene in a closed chamber for about 15 hr. The chlorophyll starts to disappear when the fruit is exposed to fresh air after the treatment.

Chlorophyll can also be a problem in the peel of late maturing Valencias. In this case, the peel generally attains a good color in the winter but during the following spring and summer, regreening, starting usually at the stem end, takes place. This is an especially important problem in California where the Valencia crop is carried over into the late summer and fall. Eaks (23) noted that ethylene accelerated the loss of chlorophyll of regreened Valencia but even after 8 days exposure to 10 ppm

considerable chlorophyll remained. This researcher questioned the practicality of degreening regreened Valencia oranges.

Degreening and Disease

There are certain problems connected with the use of ethylene for degreening. Fruit treated with ethylene during postharvest has a greater amount of decay than untreated (24). Anthracnose in Robinson tangerines became progressively worse with increasing concentrations of ethylene up to 50 ppm during degreening. The anthracnose fungus was shown to survive on the surface of tangerines in the form of appressora which, in most instances, did not produce infectious hyphae until stimulated with ethylene. Using the same cultivar, Barmore and Brown (25) found that spraying with ethephon, an ethylene-producing compound, 5 to 7 days before harvest significantly reduced the incidence of anthracnose. Control was attributed to accumulation of ethylene in the internal portion of the fruit which induced physiological changes required for development of resistance. Orange-colored fruits are more resistant to the disease than pale yellow or green colored fruit. The spraying with ethephon brought about some color changes while the fruit was still on the trees.

Carotenoids in Peel

The bright orange and yellow colors of citrus peel are due to the lipid soluble carotenoids. The more common ones in peel and juice are shown in Figure 1. Some of the first studies on citrus carotenoids were by the European workers, Zechmeister and Tuzson, in 1931 (26, 27, 28). They isolated β -carotene, cryptoxanthin, lutein, zeaxanthin, β -citraurin, and probably violaxanthin. In 1952, Natarajan and MacKinney (29) found phytofluene and α , β , and zeta carotenes. These studies were followed by those of Curl, who between 1953 and 1967 reported the presence of some 74 carotenoids in several citrus cultivars. A list of these can be found in a review by Stewart and Wheaton (30). Yokoyama and Vandercook (31) found 35 carotenoids in lemons; Gross *et al.* (32) isolated 57 pigments from Shamouti orange juice. Subbarayan and Cama (33) reported 19 in the Nagpur orange. In a recent count (34), approximately 115 carotenoids have been reported in citrus or about one-third of all those known to occur in nature (35). It is important to understand some of the reasons why such large number of carotenoids have been reported in only one genus of fruit. Carotenoids are generally unstable and have some unusual properties. Although they are visible in extremely small quantities, they are deceptively difficult to separate and identify. Finally, the lack of sophisticated techniques by researchers has undoubtedly led to questionable identification of many carotenoids. The carotenoids

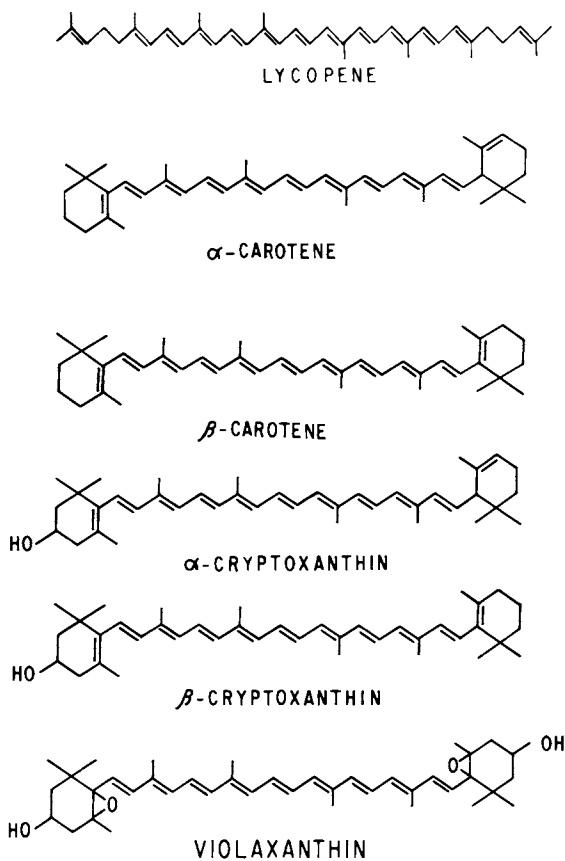


Figure 1. Some common C-40 carotenoids found in citrus peel and juice

in citrus consist of those having 30 and 40 carbon atoms. They are highly unsaturated compounds from which the chromophores are derived. The carotenes are hydrocarbons and the xanthophylls contain oxygen in hydroxyl or aldo groups or epoxides.

Isomer and Artifact Formation of Carotenoids

Carotenoids are sensitive to heat, light, acid, oxygen and chemicals. They may react in various ways during the process of isolation. The most important change is the formation of cis-isomers. Lycopene is said to form 1056 isomers (36), each with different properties. In some cases, cis-isomers separate more easily than carotenoids that differ by rearrangement of a double bond, i.e., zeaxanthin and lutein. However, a mixture of isomers can often be identified by their failure to crystallize and to give sharp separations on chromatographic columns.

The most difficult pigments to separate in citrus are antheraxanthin and violaxanthin. These carotenoids contain one and two epoxides, respectively. In the presence of a trace of acid, the 5,6-epoxides change to stable 5,8 furanoid rings. During isolation it is common to have many combinations, such as the 5,6 trans with many cis-isomers, conversion of one epoxide to the 5,8 ring with many isomers and conversion of two epoxides to the furanoid form with numerous isomers. In practice, there is usually a mixture of all of these. It has not been possible to determine which occur in nature and which are artifacts formed during isolation. The isomers of these two carotenoids are so numerous in processed orange juice that we have been unable to separate them. In our studies with fresh orange juice or peel, the main isomer of both violaxanthin and antheraxanthin has, in each case, been a cis form. For each pigment, the visible absorption spectra showed a very low cis peak suggesting the bending took place near the ring. These cis-isomers could readily be crystallized. During crystallization, about 10% of the trans was formed. However, this is to be expected since the equilibrium is in the direction of the trans isomer. Isomers were readily formed on chromatographic columns, and length of time on the column was important (37). Isomerization caused in this manner could be partly overcome by use of the antioxidant BHT in the solvent system. Chromatographic column packings can induce other changes. Rodriguez *et al.* (38) found α -carotene, β -carotene and lycopene underwent hydroxylation when passed through a column containing MicroGel C. This packing has been used to separate and identify citrus carotenoids.

Other problems in identification are the formation of artifacts during saponification. Carotenoids in citrus occur mainly as esters. For ease of separation, they are saponified with a strong base to their alcohols. During this reaction, traces of

acetone or other ketones will react with the aldo carotenoids to form artifacts (39).

Identification of Major Carotenoids in Orange and Tangerine Peel

Studies using HPLC (37, 40, 41) would suggest there are fewer carotenoids in citrus than have been reported. The single most important one in the peel of oranges and tangerines is β -citraurin, a reddish-orange carotenoid. Others include β -cryptoxanthin, zeaxanthin, lutein, antheraxanthin, violaxanthin and to a lesser extent β -carotene and β -apo-8'-carotenal. The carotenes do not contribute very much to the color of the peel per se.

Quantitation of Citrus Peel Carotenoids

There is very little quantitative information on the amounts of various pigments in the peel. Most researchers have reported on percentage of carotenoids based on the total carotenoids. Total carotenoids were determined using β -carotene as a standard. More recently, HPLC techniques have been used. These methods are more reproducible and it is possible to measure each carotenoid based on its extinction co-efficient. The data in Table I was obtained in this manner. About 12 fruit were sampled at random from the outside of the trees. A total of 30 discs of peel were taken for a sample using a No. 11 cork borer, 16 mm in diameter. The extraction procedure (39) and the chromatographic method (40) have been reported previously. Rather than measure an approximation of the "total carotenoid", it is better to measure reflectance with a colorimeter, which is closely related to visual color (42). Color measurements made using a Hunterlab D25 Color Difference Meter, measures reflected light using a L,a,b system that gives units of approximately visual uniformity throughout the color solid. The "a" value corresponds to a red-green scale on which red colors are positive and green values negative. The "b" value corresponds to a yellow-blue scale on which yellow values are positive (Figure 2). The a/b values correlated well with the USDA color standards.

Carotenoids and Peel Color

The values given in Table I of the major carotenoids in the peel of seven cultivars are useful in understanding the quantitative changes that take place during the coloring process. However, the understanding of the contribution of each carotenoid toward the total color of the peel remains entirely qualitative. For example, violaxanthin occurs in the largest amounts of any pigment in the peel in most cultivars. It is yellow and is important in the color of lemons and grapefruit. Yet, increasing the amounts in orange or tangerine peel hardly

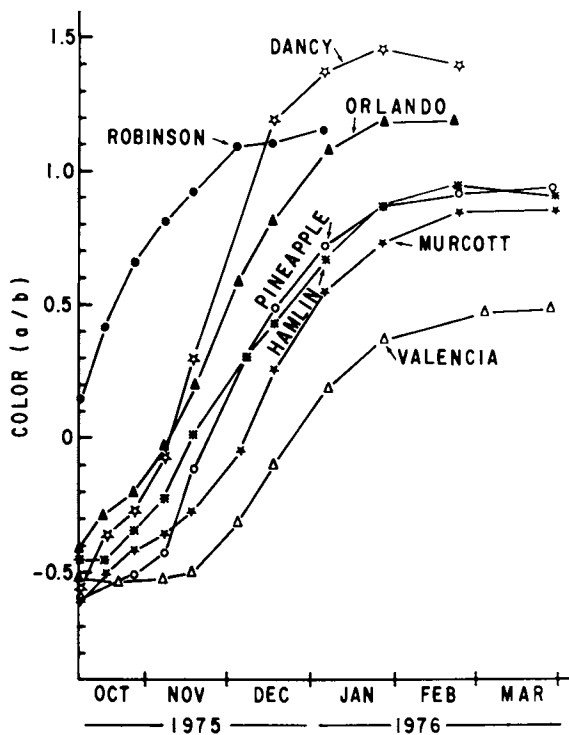


Figure 2. Effect of time of sampling and cultivar on the a/b color ratios of citrus peel. The a/b ratios are negative for green, approximate zero for yellow, and are positive for orange through red.

helps the orange and red color of these cultivars. On the other hand, β -citraurin, a reddish-orange pigment and β -cryptoxanthin, an orange carotenoid, contribute greatly to the desired colors of these fruit.

30-Carbon Carotenoids

Undoubtedly, the most interesting group of carotenoids in citrus peel are those that contain 30 carbon atoms (Figure 3). Until recently, these were considered to include β -citraurin and β -apo-8'-carotenal. There has been speculation that β -citraurin was a breakdown product of zeaxanthin (43). However, with the discovery of two other related compounds, β -citraurinene (44) and β -citrauroil (45), it was suspected there may be a C-30 carotenoid metabolic pathway. Later, some additional evidence was added to this belief when another C-30 compound (C-30 phytoene) was tentatively identified in a peel extract with the use of a high resolution mass spectrometer. The fragment found was one with a mass of 408.3763. Mass calculated for C-30 phytoene $C_{30}H_{48}$ was 408.3756. Taylor and Davis (46) reported a mass of 408.375995 for C-30 phytoene in bacteria. If subsequent studies demonstrate a C-30 metabolic pathway in citrus peel, this could open up a new approach toward improving the peel color of oranges and tangerines.

Ethylene in Carotenoid Synthesis

Ethylene has been used for many years to reduce the chlorophyll content of citrus peel; however, more recently it was found equally important in bringing about the accumulation of certain carotenoids. Stewart and Wheaton (47) found the major red pigment in tangerine and orange peel, β -citraurin, could be greatly increased during postharvest storage by use of ethylene. Initially, it was believed that β -cryptoxanthin increased from the use of ethylene (47) since this pigment was isolated from a chromatographic band associated with an increase in color following exposure of the fruit to ethylene. Subsequently, the band was found to be a mixture of β -cryptoxanthin and a new C-30 carotenoid, β -citraurinene (44). The new carotenoid and not β -cryptoxanthin was found to increase simultaneously with β -citraurinene during color formation. This increase of C-30 carotenoids in the peel of citrus was not only dependent on ethylene but also on temperature. When the fruit was stored at 30°C, virtually no β -citraurin was found but at 25°C, 20°C and 15°C the red carotenoid increased progressively. At lower temperatures, decreasing amounts of ethylene were required to produce maximum color (9). At 15°C significant amounts of color developed without the addition of ethylene; presumably, this was caused by the endogenous ethylene in the fruit. Optimum ethylene concentrations varied from 0.1 to 1 ppm at 15°C to 10 ppm at 25°C. During the postharvest coloring

Table I. Carotenoid Content (in $\mu\text{g}/100 \text{ cm}^2$) of Peel from Seven Citrus Cultivars

Cultivar	Sampling dates								
	10-7	10-27	11-19	12-19	1-7	1-28	2-23	3-30	
				α-Carotene					
Hamlin	19 ± 1.0	12 ± 0.0	3 ± 0.0	3 ± 1.0	1 ± 0.0	1 ± 0.0	1 ± 0.0	4 ± 1.0	
Pineapple	54 ± 1.0	36 ± 1.0	4 ± 2.0	2 ± 0.0	2 ± 0.0	2 ± 0.0	2 ± 0.0	5 ± 1.0	
Valencia	26 ± 0.0	47 ± 1.0	37 ± 1.0	11 ± 0.0	t	t	t	t	
Robinson	11 ± 0.0	4 ± 0.0	2 ± 0.0	3 ± 0.0	t	t	t	t	
Dancy	70 ± 0.0	46 ± 2.0	1 ± 0.0	7 ± 0.0	t	t	t	t	
Orlando	18 ± 0.0	7 ± 0.0	3 ± 1.0	2 ± 1.0	t	t	t	t	
Murcott	57 ± 1.0	19 ± 2.0	10 ± 0.0	3 ± 0.0	t	t	t	t	
				β-Carotene					
Hamlin	28 ± 0.0	53 ± 2.0	17 ± 1.0	5 ± 0.0	3 ± 0.0	12 ± 0.0	19 ± 1.0	14 ± 2	
Pineapple	52 ± 1.0	57 ± 1.0	43 ± 1.0	10 ± 1.0	6 ± 1.0	23 ± 0.0	21 ± 1.0	20 ± 1.0	
Valencia	72 ± 1.0	155 ± 5.0	120 ± 0.0	67 ± 1.0	22 ± 4.0	18 ± 1.0	22 ± 1.0	37 ± 2.0	
Robinson	14 ± 0.0	13 ± 0.0	13 ± 1.0	14 ± 1.0	14 ± 1.0	14 ± 1.0	14 ± 1.0	14 ± 1.0	
Dancy	44 ± 2.0	115 ± 5.0	52 ± 1.0	20 ± 3.0	17 ± 0.0	t	t	t	
Orlando	54 ± 2.0	14 ± 0.0	12 ± 1.0	4 ± 1.0	4 ± 1.0	7 ± 1.0	15 ± 0.0	24 ± 0.0	
Murcott	84 ± 4.0	42 ± 1.0	78 ± 5.0	13 ± 0.0	4 ± 0.0	38 ± 0.0	33 ± 0.0	33 ± 0.0	
				APO-8' Carotenal					
Hamlin	t	t	7 ± 1.0	6 ± 0.0	8 ± 1.0	24 ± 0.0	36 ± 2.0	17 ± 1.0	
Pineapple	t	t	2 ± 1.0	8 ± 0.0	18 ± 0.0	27 ± 0.0	29 ± 1.0	33 ± 1.0	
Valencia	t	t	t	3 ± 0.0	10 ± 0.0	11 ± 1.0	11 ± 1.0	11 ± 1.0	
Robinson	16 ± 0.0	86 ± 8.0	86 ± 0.0	94 ± 1.0	104 ± 6.0	110 ± 0.0	160 ± 0.0	160 ± 0.0	
Dancy	t	29 ± 7.0	48 ± 3.0	53 ± 2.0	64 ± 11.0	20 ± 1.0	26 ± 1.0	26 ± 1.0	
Orlando	t	t	7 ± 0.0	9 ± 0.0	12 ± 1.0	78 ± 8.0	145 ± 5.0	103 ± 7.0	
Murcott	t	t	t	18 ± 5.0	22 ± 5.0	78 ± 8.0	145 ± 5.0	103 ± 7.0	
				β-cryptoxanthin					
Hamlin	8 ± 1.0	6 ± 1.0	9 ± 0.0	26 ± 2.0	37 ± 2.0	60 ± 2.0	63 ± 2.0	110 ± 0.0	
Pineapple	4 ± 0.0	7 ± 0.0	11 ± 0.0	32 ± 6.0	76 ± 5.0	110 ± 0.0	105 ± 5.0	185 ± 5.0	
Valencia	4 ± 0.0	5 ± 0.0	13 ± 1.0	15 ± 0.0	33 ± 1.0	34 ± 1.0	46 ± 1.0	65 ± 6.0	
Robinson	86 ± 2.0	215 ± 5.0	335 ± 5.0	390 ± 0.0	380 ± 10	2320 ± 40	2665 ± 175	2665 ± 175	
Dancy	13 ± 1.0	86 ± 9.0	260 ± 0.0	960 ± 50	1270 ± 10	44 ± 2.0	52 ± 2.0	52 ± 2.0	
Orlando	4 ± 1.0	4 ± 1.0	18 ± 1.0	43 ± 2.0	73 ± 1.0	685 ± 25	1260 ± 0.0	1680 ± 40.0	
Murcott	21 ± 1.0	43 ± 0.0	71 ± 1.0	265 ± 5.0	275 ± 5.0	685 ± 25	1260 ± 0.0	1680 ± 40.0	

Table I. (Continued)

Cultivar	Sampling dates							
	10-7	10-27	11-19	12-19	1-7	1-28	2-23	3-30
				β-Citraurain				
Hamlin	t	t	17 + 2.0	47 + 2.0	105 + 5.0	255 + 5.0	210 + 0.0	185 + 5.0
Pineapple	t	t	t	65 + 0.0	145 + 5.0	245 + 5.0	175 + 5.0	160 + 0.0
Valencia	t	t	t	10 + 0.0	43 + 1.0	58 + 0.0	54 + 2.0	190 + 0.0
Robinson	85 + 5.0	300 + 10.0	465 + 15.0	665 + 45.0	755 + 25.0			
Dancy	t	54 + 2.0	140 + 10.0	545 + 50.0	700 + 10.0			
Orlando	t	9 + 1.0	64 + 0.0	205 + 5.0	445 + 5.0	320 + 0.0	485 + 5.0	
Murcott	t	t	t	57 + 2.0	80 + 0.0	215 + 5.0	285 + 5.0	215 + 15.0
				Lutein				
Hamlin	61 + 0.0	42 + 0.0	20 + 0.0	t	t	t	t	t
Pineapple	110 + 9.0	87 + 0.0	36 + 0.0	25 + 1.0	t	t	t	t
Valencia	68 + 9.0	127 + 0.0	51 + 0.0	38 + 9.0	43 + 0.0	47 + 0.0	t	t
Robinson	65 + 5.0	84 + 0.0	t	t	t	t	t	t
Dancy	120 + 4.0	130 + 1.0	101 + 0.0	150 + 2.0	130 + 5.0	170 + 27.0		
Orlando	16 + 1.0	55 + 0.0	27 + 1.0	t	t	t	t	t
Murcott	150 + 3.0	80 + 1.0	75 + 9.0	56 + 2.0	29 + 1.0	t	t	t
				Violaxanthin				
Hamlin	27 + 1.0	46 + 0.0	115 + 5.0	260 + 10.0	305 + 5.0	945 + 15.0	1090 + 20.0	1135 + 35.0
Pineapple	8 + 0.0	15 + 1.0	59 + 2.0	160 + 0.0	405 + 1.0	855 + 35.0	1235 + 5.0	1445 + 25.0
Valencia	t	12 + 0.0	8 + 0.0	120 + 0.0	265 + 5.0	540 + 10.0	1040 + 0.0	
Robinson	175 + 5.0	410 + 20	660 + 0.0	735 + 15.0	1115 + 5.0			
Dancy	90 + 0.0	305 + 15.0	465 + 25.0	1005 + 15.0	1275 + 5.0	2000 + 20.0	2705 + 55.0	
Orlando	26 + 2.0	73 + 0.0	210 + 0.0	460 + 20.0	885 + 5.0	625 + 5.0	1160 + 10.0	
Murcott	10 + 1.0	34 + 2.0	74 + 1.0	195 + 5.0	245 + 5.0	1065 + 35.0	1645 + 5.0	1505 + 15.0

period, the synthesis of carotenoids could be virtually controlled by changing temperature and ethylene concentrations (9).

Carotenoids in Juice

The carotenoids in citrus juice in comparison with those in the peel differ in two important respects. In Florida, we have found no C-30 carotenoids in juice. This is based on observations made on several hundred samples, using a variety of techniques. However, Gross *et al.* (32) reported finding citraurin in Shamouti orange juice and in Valencia orange juice (48). Secondly, our studies (49) indicate that the main carotenoids in orange and tangerine juice are α -carotene, β -carotene, zeta carotene, α -cryptoxanthin, β -cryptoxanthin, lutein zeaxanthin, antheraxanthin and violaxanthin (Figure 3). Although antheraxanthin and violaxanthin occur in the largest amounts, they are yellow pigments. β -cryptoxanthin, an orange pigment, is the main contributor to the orange color in orange juice.

In pink and red grapefruit juices the main pigment is lycopene. Ting and Deszyck (50) found 1.3 mg and 2.3 mg of lycopene in 100 g of pink and red grapefruit, respectively. They also reported the fruit to have 0.5 mg to 1.4 mg β -carotene per 100 g. Purcell (51, 52) found up to 1.8 mg lycopene and 0.49 mg carotene/100 g in juice sacs in Texas Ruby Red grapefruit. Maximum lycopene content was reached in August and September and dropped off after that time.

Very little is known regarding factors that affect the color of citrus juice. The author has used many combinations of temperature and ethylene with fruit of several cultivars. Although the peel color was affected, no changes were observed in the juice color. Recently, Houck *et al.* (53) found storage atmospheres containing 40% and 80% O₂ caused navel but not Valencia endocarp to turn a darker orange after 2 weeks of storage. This is the only work that we are aware of in which postharvest treatment influenced the juice color.

Color and Provitamin A

It is well-known that plants do not synthesize vitamin A. Also, animals can only synthesize vitamin A from β -carotene or carotenoids in which one-half of the molecule is like β -carotene. In nature, the vitamin A precursor comes either from plants or microorganisms. The most common sources of vitamin A in citrus are α - and β -carotenes and β -cryptoxanthin. In addition to the above name carotenoids, β -apo-8'-carotenal in citrus peel could be a source of provitamin A. However, the peel is not usually consumed. Provitamin A compounds are cleaved to form vitamin A aldehyde in the intestine by β -carotene 15,15'-oxygenase (Figure 4) (54). Aldehyde reductase reduces the aldehyde to the all trans-vitamin A. β -Carotene is cleaved between the 15,15' carbon

Table II. Provitamin A Content of Citrus Juice^a (49)

Cultivar	Vitamin A ^b value, International units	Daily recommended percentage ^b
Hamlin	80	1.6
Pineapple	133	2.7
Valencia	83	1.7
Robinson	1142	23
Dancy	965	19
Orlando	236	4.7
Murcott	3195	64

^a Calculations based on: β -carotene equals 1.667 International units vitamin A/ μ g, equals 100%; α -carotene, 52.7%; β -cryptoxanthin, 57%. Conversion factor from 100 mL of juice to 6 oz, 1.77 (70).

^b Values based on 6 oz of juice and calculated on the dietary daily allowance of 5000 IU.

atoms giving two molecules of retinal. α -Carotene, which consists of a β ring and an α ring, is split but only the β ring portion is active. With β -cryptoxanthin, which is the largest source of provitamin A in citrus juice, the 3-OH ring half is not active, leaving only the β -ring side. In case of α -cryptoxanthin, which like α -carotene contains an α ring inactive and a β ring which would be active provided the OH group was on the α ring. However, in case of citrus, the OH group in α -cryptoxanthin is on the 3 carbon of the β -ring (40). Violaxanthin, the carotenoid that occurs in the largest amounts in citrus juice, does not have any provitamin A activity because of substitutions on both rings.

The vitamin A content of orange juice as given by Adams (55) is about 200 International units of provitamin A in 100 g. In more recent studies (Table II) (49), orange juice was found to have the least amount of provitamin A whereas Murcott (a reputed tangor) had the highest of the seven cultivar studies. The lowest found was in the Hamlin orange with 80 International units/6 oz of juice. The highest was Murcott with 3195 International units/6 oz of juice. All samples were taken from mature fruit except Valencia. When mature, Valencia juice normally contains more provitamin A than Hamlin or Pineapple juice.

In grapefruit juice there was a wide difference between provitamin A in white and red or pink. White grapefruit juice contained approximately 8 International units of provitamin A per 100 g of juice, whereas pink and red contained 440 (55). This difference in provitamin A is due to β -carotene. Ting and Deszyck (50) showed red and pink grapefruit juice to contain 1.0 to 1.4 mg of β -carotene per 100 g of juice when the fruit was fully mature. When calculated on the value of 1.667 International units of vitamin A = 1 μ g of β -carotene, the Florida juice would have contained the equivalent of 1667 to 2334 units of vitamin A/100 g. Purcell (51, 52) reported approximately one-half this value for red grapefruit juice from Texas fruit.

Standards for Color in Citrus and Citrus Products

The United States Department of Agriculture (56), the Florida Department of Citrus (57), and the California Department of Food and Agriculture (58) have set standards for grades of fresh fruit, concentrated orange juice and other citrus products. Processed products are given one of two grades by government inspectors: U. S. Grade A and U. S. Grade B. Any product graded below this is classed as substandard. In grading citrus products, a scoring system based on 100 points is used. In case of orange juice the system is as follows:

<u>Factors</u>	<u>Points</u>
color	40
defects	20
flavor	<u>40</u>
total score	100

Orange juice with good color, having a color score of 36 to 40 points, may be classified as Grade A. Juice with good color but with a color score of 32 to 35 cannot be graded above U. S. Grade B regardless of other characteristics. Orange juice with a color score of 31 or below must be graded substandard.

Two methods are used to measure color of orange juice. The oldest of these procedures is made by comparing the color of reconstituted products if it is concentrated with the USDA orange juice color standards. These color standards range from yellow to yellow orange. A tube of juice to be graded is compared with the standard tubes using specified lighting conditions. The second method for evaluating color is by the use of colorimeters approved by the USDA. In Florida, the Department of Citrus has ruled that the Hunterlab Model D-45 Citrus Colorimeter shall be used exclusively to determine the color scores for orange juice. The Hunterlab Citrus Colorimeter was developed in 1963 for the Florida Citrus Commission. This is a reflectance instrument that measures citrus redness (CR) and citrus yellowness (CY). By means of a formula, the color score is calculated: Color score = $22.51 + (CR \times 0.165) + (CY \times 0.11)$. Color scores obtained with the citrus colorimeter have been correlated with those using the USDA color standards (59, 60).

In Florida, there are regulations on the dilution of concentrated orange juice that can substantially affect the color score. For example, juice concentrated to 45°Brix must be reconstituted to 12.8°Brix before a color determination is made. That concentrated to 42°Brix must be reconstituted to 11.8°Brix. Concentrated orange juice for manufacturing is reconstituted to 12.3°Brix.

Grapefruit juice standards like those for orange juice allocate 40 points out of 100 for color. However, the similarity of scoring the two juices ends at that point. There are no instrumental methods in use for determining the color of grapefruit juice. Rather ambiguous terms are used to describe color of grapefruit juice such as U. S. Grade A or U. S. Fancy which requires that the juice have "very good color," meaning that it is bright and typical of fresh extracted grapefruit juice. It may be pale yellow to very slightly amber, typical of white grapefruit juice or slightly red typical of red or pink grapefruit juice. Obviously, it is difficult to enforce these regulations. Standards for color of all other processed citrus products are equally as subjective. This does not infer that

there are not satisfactory objective techniques for determining the color of processed citrus other than orange juice. Huggart and Petrus (61) and Huggart *et al.* (62) found the visual color changes in white and pink grapefruit juice were closely associated with Hunterlab Citrus Colorimeter measurements. Previously, Ting and Deszyck (50) had shown a close correlation between Hunter a/b values and lycopene content in pink and red grapefruit.

Color Standards for Fresh Fruit

In Florida, color standards for fresh fruit are based entirely on the color of the peel (57). Subjective means are used to describe the color. For example, some of the USDA color grade standards for Florida oranges and tangelos are: U. S. Fancy - Well-colored, meaning that the fruit is yellow or orange in color with practically no trace of green color. U. S. No. 1 Bright - Early and midseason varieties must be fairly well-colored. This means the yellow and orange colors predominate over the green color. An aggregate area of green color may not exceed the area of a circle 1 inch in diameter. For Valencias and other late varieties not less than 50% by count shall be fairly well-colored and the remainder reasonably well-colored. Reasonably well-colored means the yellow or orange color predominates on at least two-thirds of the fruit surfaces in the aggregate. There are still other grades with descriptive color requirements.

In California, the color requirements are much different (58). The color of the peel is tied in with the juice quality, namely, the ratio which is the soluble solids divided by the acid content. Fruit having a bright peel color can be marketed with poorer juice quality (lower ratio) than that with poor peel color. This is a case where the best looking fruit may not be the best tasting.

Color Enhancement in Fresh Fruit

In Florida, it is not uncommon in the fall for the internal part of the fruit to be palatable at maturity but the peel to be mostly green with a color break. After the fruit has passed through the degreening process, it has a yellow appearance. The pigments are mainly lutein, violaxanthin, and neoxanthin. This fruit may then be passed through a color-add tank containing Citrus Red No. 2 (63). The fruit is then thoroughly rinsed and a maximum of 2 ppm residue is permitted. The operation is time and temperature dependent and takes place under the supervision of government inspection (57). Color-add is permitted only on oranges, Temples and tangelos and the fruit must have higher internal quality than natural colored fruit.

Color Enhancement of Orange Juice

No artificial coloring material is permitted to be added to orange juice. Almost one-half of the orange juice processed in Florida comes from early and midseason varieties. In many cases this juice does not have a color score of 35, the minimum required for Grade A. Color standards are met by blending. This is done in several ways: (a) Early and midseason orange juice is stored and blended with fruit from the late-maturing Valencia fruit which has more than sufficient color to meet Grade A standards; (b) Valencia juice may be stored until the following season when it is blended; (c) Tangerine and hybrid juice which has a bright color may be blended but only to a maximum of 10%, according to FDA standards of identity for orange juice; (d) Finally, another source of color for orange juice in Florida is the use of imported juice selected for bright color.

In the laboratory, color concentrates have been made from orange peel for the purpose of improving the color of juice. Ting and Hendrickson (64) extracted orange peel with acetone and hexane. The peel extract from 1226 kg of pineapple oranges was sufficient to increase the color score in 3785 l of juice from 37 to 38. In other studies (65), it was pointed out that 1 g of color concentrate from either Pineapple or Valencia peel in 2 liters of juice increased the color score from 35 to 40. In similar studies, Berry *et al.* (66) reported on the extraction of carotenoids from citrus peel with hexane.

Another approach to the same problem was to concentrate the chromoplasts of deeply colored tangerine juice. Barron *et al.* (67) and Barron and Metcalf (68) did this by means of centrifuging juice and collecting the colored pellets. It has been suggested that the regular run Valencia orange juice be split and half be used for color extraction. The chromoplasts would be used to improve the color of juice from early and midseason varieties. The juice left from the chromoplast extraction would be mixed with the remaining half of the Valencia juice, giving a color score sufficiently high to meet Grade A standards. Naturally occurring coloring material obtained by this means could be added to orange juice without any adulteration being considered a color addition (69). None of the color concentrates are presently being used in orange juice.

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Relationship of Citrus Enzymes to Juice Quality

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The quality of citrus fruit represents the sum total of fruit development--fruit set, growth, tissue differentiation and ripening on the tree. During this development period compounds are formed that are responsible for the color and flavor of the ripe fruit. Many of these compounds have been identified, and research is now directed toward identifying the enzymic reactions that regulate their biosynthesis through metabolic pathways. Stewart (1) recently reviewed the carotenoid pigments identified as present in citrus and assessed their contribution to citrus color. Reported with that review in the same journal was the mode of action of bioregulators in controlling carotenoid biosynthesis through enzyme inhibition (2). Many terpenoids, aliphatic esters and aliphatic aldehydes in citrus fruit have been identified, but the pathways for their biosynthesis have not yet been determined (3). Bruemmer et al. (4) recently reviewed the working hypotheses on the mechanism for regulating the biosynthesis of citrus acids and concluded that supportive data were inadequate to identify the enzyme reactions that regulate acid metabolism in citrus.

The quality of extracted citrus juices depends on enzyme reactions that occur not only in the fruit during the development period, but also in the juice during processing. When juice is extracted from citrus fruit, enzymes are released from their normal restraint in the cell. Several of these enzymes catalyze reactions that adversely affect taste and appearance of the juice. Unless the reactions are controlled, the juice products will not meet the standards of quality set up by the USDA Food Safety and Quality Service. The two reactions of commercial importance are the hydrolysis of pectin to pectic acid, which clarifies juice, and the lactonization of limonoic acid A-ring lactone to the bitter compound, limonin. Research efforts to identify and characterize the reactions, to isolate and purify the enzymes, and to develop methods to control the reactions are described in this review.

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Pectinesterase (E.C. 3.1.1.11)

Early in the development of the citrus juice processing industry, clarification of bottled and canned citrus juices and citrus beverages was recognized as resulting from the action of pectic enzyme(s) that had not been destroyed by heat pasteurization (5). Investigations of cause and prevention of clarification and gelation in citrus juices and their concentrates were stimulated by the rapid expansion of the frozen-orange concentrate industry in the late forties. These investigations were thoroughly reviewed by Joslyn and Pilnik (6). During the past twenty years some progress was made on purification and mode of action of pectic enzymes (7). More recently, multiple forms of pectinesterase (PE) that differ in kinetic properties and temperature stability have been isolated (8). Some of the irregularities in heat inactivation of PE and juice cloud stability can be attributed to the actions of the different forms of PE. The more recent research on PE and the older work on citrus juice stabilization are reviewed to relate PE to the problem of juice quality.

Juice Clarifying Enzyme. One of the earliest reports on a clarifying and clotting enzyme in orange juice was by Cruess (9). He reported that fresh orange juice formed a jelly-like suspension a few hours after expression, and that after a few days the suspended matter coalesced and settled, leaving a clear supernatant liquid. He speculated that heating the juice to 85°C (185°F) destroyed enzyme(s) and prevented the juice from clearing. Joslyn and Sedky (10) showed that clarification of citrus juices was always accompanied by decomposition of pectic substances and used the rate of clarification as a measure of the activity of the pectic enzymes. They (11) found that the rate at which heat pasteurized citrus juices cleared varied inversely with both temperature and duration of the heat treatment. They showed that heating orange juice to 80°C (176°F) at pH 4 for 1 min inactivated the clearing enzyme and that inactivation was more rapid at pH 2.5 than at pH 4.0. Stevens (12) elaborated on the pH-temperature relationship in his patent for stabilizing citrus juice products by setting forth four specific ranges of minimum temperatures for short time (0.1 to 3 min) heating of juices at pH 2.2 to 3.79. Also he speculated that citrus juices contain two cloud-coagulating enzymes of different thermostabilities. One enzyme, most active at low pH and temperature, appeared to be destroyed by heating the juice at 65 to 70°C (149 to 158°F). The second enzyme, most active at pH 3.0 to 3.3 and about 35°C (95°F), appeared to require heating to 88°C (191°F) for inactivation (12). Stevens (13) described a rapid test for pectic enzymes in citrus juice. It involved adding pectin under controlled conditions of temperature and sample preparation, and measuring the time required for flocculation. Stevens, and coworkers (14) further elaborated on the patent work (12, 13). They produced a trend curve of the

minimum temperatures required to satisfactorily inactivate pectic enzymes in natural strength citrus juices of different pHs. Their data indicated that the pulp content of juice was the most important factor determining the rate and completeness of flocculation. Pulpy juice required higher temperature for cloud stabilization. They also found that the pasteurization conditions necessary to stabilize natural strength or 42°Brix concentrated orange juice for frozen storage (-23.3°C; -10°F) was about 65°C (149°F) for 1 min. They reported that these conditions coincided with the minimum conditions necessary to destroy spoilage organisms.

A quantitative objective measurement of citrus juice turbidity was used by Loeffler (15, 16) to show that pectic enzyme changes occurred so rapidly after the juice was reamed from the fruit that at least a partial coagulation of the cloud occurred before the juice could be screened, deaerated and heated to a pasteurization temperature. He showed that juice turbidity was increased by flash-pasteurization and also by homogenization of the juice before pasteurization. Loeffler (15, 16) presented data on turbidity of flash-pasteurized citrus juices (heat exposure for 16 to 18 sec) after storage at several temperatures. He found that "samples pasteurized at 91°C (196°F) lost their cloud when stored at 35°F (95°F) but others pasteurized at 93-95°C (199-203°F) retained their cloud almost indefinitely".

Extraction and Identification. Identification of PE as the clearing enzyme in citrus juices progressed rapidly after MacDonnell et al. (17) reported on cation requirement for extraction and solubilization of the enzyme from various portions of the fruit. PE was assayed by the method introduced by Kertesz (18) and modified by Lineweaver and Ballou (19). The method involved measuring the rate at which the methyl ester groups in the pectin molecule are hydrolyzed by titrating the free carboxyl groups with 0.1N NaOH as they are formed. One unit of PE was defined as the amount of enzyme which will hydrolyze 1 meq carboxyl groups per min from a 0.5% solution of pectin in 0.15M NaCl at pH 7.5, 30°C (86°F). McDonnell et al. (17) showed that the enzyme extracted from orange flavedo had a rather broad pH optimum at about 7.5 in 0.15M NaCl, but that the enzyme required high ionic strength solutions at lower pH to be optimally active. For 0.15M CaCl₂ the plateau of optimum activity extended from pH 4.0 to 8.5 but activity was only about 50% of the level of activity found for 0.05M CaCl₂ at pH 7.5. The increased activity caused by cation was greater at pH 5 than at pH 7.5 with 0.05M CaCl₂.

Occurrence and Distribution. PE was found associated with structural elements of the orange. McDonnell et al. (17) reported no activity in filtered orange juice, but found 58, 44 and 28 PE units per kg wet tissue in flavedo, albedo and cell sacs respectively. Working with four varieties of Florida oranges and Dancy tangerine, Rouse (20) showed that juice sacs had the highest

activity and centrifuged (not clarified) juice the least. The rag (segment wall tissue enclosing the juice sacs) had the next highest activity followed by the peel (flavedo and albedo). In grapefruit he found that juice sacs had the highest activity but that albedo and flavedo had more activity than the rag. The distribution of PE in lemon and lime tissues was somewhat different from that in orange or grapefruit. Rouse and Atkins (21) showed that lemon and lime peel (flavedo plus albedo) had the highest activity followed by juice sacs and rag in that order. Previously, Rouse (22) found that juice samples prepared to contain increasingly higher amounts of pulp (juice sacs), also had correspondingly higher PE activities. Rouse, Atkins and Huggart (23) showed that PE activity in orange juice was directly proportional to pulp content.

Orange PE was shown to be bound to cell walls as an enzyme-substrate complex with pectin (24). The solubilization of PE (pH 7.5 and 0.15N NaCl) and de-esterification of cell wall pectin were similarly temperature dependent. After complete de-esterification of the cell wall pectin, an equilibrium was established between bound and free PE in the extraction medium. At the pH of juice (4.5 and below) the enzyme bound to cell walls was not solubilized unless soluble pectin was added. The bound enzyme was inactive at pH 4, whereas the free soluble enzyme was about 20% more active at pH 4 than at the optimum pH (7.5).

Assays for PE. Because PE hydrolyzes pectin to pectic acid and methanol, the enzyme concentration can be assayed by measuring the rate at which free carboxyl groups or methanol is released from the substrate. Kertesz (18) titrated the free carboxyl groups as they were formed by the action of the enzyme on pectin. He used methyl red to indicate the pH (6.2) and added 0.1N NaOH at frequent intervals to maintain the pH relatively constant for 30 min. The pH meter replaced the use of indicators in subsequent modifications (17, 19). Current procedures use automatic pH titrators to titrate alkali at constant pH (25). A blank is used to correct for the consumption of alkali due to its reaction with atmospheric CO₂, or the reaction solution is protected from CO₂ with a blanket of N₂.

The broad pH optimum for PE was used by Somogyi and Romani (26) to devise a rapid assay based on the rate at which the pH of unbuffered 1% pectin in 0.2M NaCl changed from 7.0 to 7.5 after the addition of the enzyme. At low enzyme concentration the reaction rate was first order for the first 1-min interval, and reproducibility was within ± 5%.

Methanol can be determined colorimetrically but must usually be distilled from the reaction mixture before being analyzed. Wood and Siddiqui (27) described a simple and precise spectrophotometric method for measuring methanol in an assay for PE. The method involved permanganate oxidation of methanol to formaldehyde and reaction of the formaldehyde with pentane-2,4-dione. The color reaction was developed directly in the PE reaction mixture without interference from the pectin. However, Termote et al. (28)

increased the sensitivity and reproducibility of the method by developing the color with methanol distilled from the PE reaction mixture.

Gas chromatographic (GC) analyses are more sensitive and reproducible than colorimetric analyses. Gessner (29) developed a method for reacting nitrous acid with the alcohols in extracts of tissue homogenates and then analyzing the head space by GC. The derivatives formed have higher vapor pressures than the alcohols so that sensitivity of head space analyses is increased accordingly. Gessner claimed good reproducibility of values with concentrations of methanol at 1 mg/l. The derivatization method was used to assay PE in plant tissue (30). A probable error of 2% was claimed, and sensitivity was 3 mg/l.

The most sensitive assay method for PE activity is to use, as substrate, pectin esterified biologically (31) or chemically (32) with ^{14}C methanol. In studies by Gessner (29) and Bartolome and Hoff (30) the substrate and enzyme were precipitated from the reaction mixture with acidified ethanol or TCA before activity of the ^{14}C methanol in the supernate was determined. The radioactive assay method was about 100 times as sensitive as the titrimetric method for PE activity (32).

The radioactive method may be used when the titration method is not applicable, e.g., when the pH of the reaction is near the pK of pectin (about 4.0), or when the reaction rate is low because of limited amount of enzyme or substrate. However, the automatic titration method when applicable, is advantageous because the reaction can be monitored continuously and any deviations from linearity readily recognized.

Purification. Orange PE was isolated initially from Navel orange flavedo by extraction with a borate-acetate buffer at pH 8.2 and then precipitation from the extract with $(\text{NH}_4)_2\text{SO}_4$ (17). Later MacDonnell et al. (33) used $(\text{NH}_4)_2\text{SO}_4$ to fractionate the flavedo extract (pH 7) and adsorbed the 40 to 80% saturation fraction on filter paper pulp (1-2 g/l). PE was extracted from the paper pulp with 0.1M NaCl and transferred to a Celite 505 column (0.4U PE/g). The column was first washed with 0.025M Na_2HPO_4 , and the PE was then eluted from the column with 1.0M NaCl containing 0.025M Na_2HPO_4 . The enzyme was precipitated from the extract with $(\text{NH}_4)_2\text{SO}_4$ (90% sat., pH 7), solubilized, dialyzed and then transferred to a Ducil column (0.75U PE/g). The column was washed with 0.025M Na_2HPO_4 , and PE was eluted from the column with 1.0M NaCl containing 0.025M Na_2HPO_4 . The eluate was dialyzed and freeze-dried; and its powder refrigerated. This procedure purified orange PE over 100-fold on a total N basis.

More recently, Manabe (34) purified PE from Citrus natsudaïdai fruit by chromatography on a DEAE-cellulose column followed by Sephadex G-100 column adsorption of the active fraction. The final preparation was homogeneous as determined by disc electrophoresis and was 460 times as active as the original extract on a protein basis.

Specificity. Orange PE hydrolyzed methyl and ethyl esters of polygalacturonans, but at optimal pH, the rate on ethyl esters was about 10% faster than that on the methyl esters (33, 35). At pH 4 the rates were about equal (35). Glycyl and glyceryl esters of polygalacturonans were not hydrolyzed by orange PE (35, 36). Non-galacturonate esters (over 50 tested) were not hydrolyzed by the purified PE preparation of MacDonnell et al. (33). Purified citrus PE did not hydrolyze methyl esters of digalacturonic or trigalacturonic acid but readily hydrolyzed polymeric units of 10 or more (37). So far, the minimum chain length of the oligogalacturonan methyl esters required for PE action has not been determined. Partial reduction of the galactopyranosiduronate structure by NaBH_4 decreased PE action markedly, indicating specificity of the carboxyl group for enzyme reaction (38).

Activation-Inhibition and Function In Vivo. When 0.15M NaCl was added to an orange PE-pectin reaction mixture at pH 7.5, activity was increased 5-fold, and at pH 5 it was increased 100-fold (17). As explained by Lineweaver and Ballou (19), NaCl caused the apparent activation by freeing the enzyme from the inactive ionic complex (pectin-carboxyl). They showed that at pH 5.7 pectic acid inhibited alfalfa PE activity 55% in 0.015M NaCl but only 17% in 0.2M NaCl. At pH 8.5 pectic acid inhibited PE activity only 9% in 0.015 NaCl. They concluded that the stimulation of activity by cations at low pH (17) did not show that cations were essential for activity, but, rather, that cations function by preventing product inhibition, which is greater at low pH.

Termote et al. (28) studied product inhibition of orange PE as an approach to stabilizing cloud of orange juice. Both chemically and enzymically prepared hydrolysates of pectic acid with an average degree of polymerization of 8 or higher inhibited PE activity at pH 5 in 0.1M NaCl. Unhydrolyzed pectic acid showed the greatest inhibition. No inhibition was observed when the ionic strength of the reaction mixture was increased to 0.5M NaCl. Pectic acid hydrolysates with degree of polymerization of 8 to 15 were effective in extending the period of cloud stability in PE active juices by a factor of 3 to 5 but did not prevent the juices from clarifying eventually.

Versteeg (8) speculated on the function of PE in vivo. He noted the high activity of PE in citrus fruit compared to the amount of available pectin. The fruit contain sufficient activity to deesterify the pectin to low methoxy pectin in 10 min at optimum pH. He suggested that the methyl transferase found by Kauss and Hassid (39) to esterify pectic acid to pectin in mung bean shoots and to be located in a lipid-membrane complex (31) functioned as pectinesterase after the lipid membranes were destroyed and the environment changed. However, no definitive experiments to establish the role of PE in fruits were reported.

Isoenzymes. Multiple forms of citrus PE were reported by Evans and McHale (40) and Versteeg et al. (41). PE was purified from West Indian limes and Navel oranges by fractionation of the whole fruit extracts with $(\text{NH}_4)_2\text{SO}_4$ (40-65%), adsorption and elution from Sephadex G-75 columns (40). The PE active fractions were combined and concentrated before separation into two active PEs on the basis of their elution volume from a DEAE Sephadex A-50 column. Orange PEI (OPEI) and lime PEI (LPEI) had the same elution volume; also OPEII and LPEII had the same elution volume. A higher concentration of NaCl was required at all pH values for optimum activity of OPEI and LPEI than of OPEII and LPEII. When the component parts of oranges were separately analyzed chromatographically with DEAE-Sephadex A-50, OPEI was detected only in the peel, whereas OPEII was identified in juice sacs and section walls (40).

Versteeg et al. (41) purified extracts of Navel orange pulp and peel by $(\text{NH}_4)_2\text{SO}_4$ fractionation (30 to 75% saturation) and chromatography on Bio-Gel P-100. The enzyme preparation eluted from Bio-Gel P-100 was separated into PEI and PEII by chromatography on cross-linked pectate (42). Each PE was further purified by chromatography on CM Bio-Gel. PEI did not bind as strongly as PEII to either the cross-linked pectate or CM Bio-Gel. The activity of the purified PEI and PEII combined was about 26% of the activity of the crude extract; and PEI had about twice the activity of PEII. Both enzymes had molecular weight of 36,200 as determined from their mobility based on dodecyl sulfate electrophoresis. PEI had optimum activity at pH 7.6 and PEII at pH 8.0. Also, PEI was relatively more active at low pH values. PEI had low affinity for pectin and was weakly inhibited by polygalacturonate. PEII had high affinity and was strongly inhibited. Rombouts et al. (42) synthesized cross-linked pectate with 0.46 degree of cross-linking and was able to get strong binding of both PEI and PEII to the pectate. Through PE activity measurements on methyl-esterified cross-linked pectate they concluded that binding of the enzymes to the pectate matrix involved biospecific affinity as well as ion-exchange effects. Versteeg (8) isolated a third isoenzyme of Navel oranges and found that it was more heat stable than either PEI or PEII and had an isoelectric point similar to that of PEI (pH 10.05). The specific activity of the heat stable fraction was low compared to that of PEI or PEII and was not improved much by Bio-Gel P-100 chromatography. The molecular weight of this third PE isoenzyme was estimated to be 54,000 and was called high molecular weight (HM-PE) by the author (8). Cross-linked pectate chromatography separated HM-PE into three fractions. HM-PEII contained about 85% of the activity of the original HM-PE and was purified about 20-fold by cross-linked pectate chromatography (8). Thin-layer pH gradient electrophoresis of crude PE extracts from different citrus fruit (oranges, lemon, grapefruit and tangerine) gave good separation of PEI, PEII and HM-PEII and revealed activity peaks corresponding to a total of 12 forms of PE. However PEI and PEII accounted for more than 80% of the activity in oranges (8).

Mode of Action of PEI and PEII. Versteeg (8) measured reaction rates of PEI and PEII with pectins presaponified so that degree of esterification (DE) ranged from 95.6 to 32%. He found that maximum velocities (V_{max}) for the reactions with all the substrates were about the same. However, the affinity of PEI and PEII to the substrates increased dramatically as the DE of the pectins decreased. Affinity of PEII for pectin was almost 150-fold greater for pectin with DE of 32% than of 95.6%. The log of affinity correlated with the log of percentage free carboxyl groups in the pectin for both PEI and PEII even though affinity of PEII was much larger than that of PEI for the pectins. The slopes of the correlation equation were 2.08 for PEI and 2.03 for PEII, indicating that doubling the number of free carboxyl groups gave a four-fold increase in affinity. Versteeg (8) calculated the percentage of possible units that had two carboxyl groups for pectins with DEs of 95 to 32%. He found that log % units with two carboxyl groups in some fixed arrangement correlated (slope equals 2) with the log % free carboxyl groups in the pectin. Because PE is inhibited by galacturonic acid oligomers with degree of polymerization of 8 or more (28) he concluded that a substrate with two free carboxyl groups separated by six monomers was required for the optimal enzyme-substrate complex.

Versteeg (8) found that PEI and PEII were similar in the products they cleaved from pectin with DE of 95.6%. Separation of reaction products after this substrate was de-esterified to DE of 60% showed that about 30% of the substrate was not acted on by the enzyme. In contrast all the ester bonds in pectin with DE of 95.6% could be hydrolyzed by alkali.

PEI and PEII did not completely de-esterify pectins with DE ranging from 95.6 to 32%. The lower the initial DE of the substrate, the lower was the DE of the final product of the reaction. However, none of the products from PEI and PEII reactions with pectins (DE 95.6 to 32%) had DEs of less than 11%. Solms and Deuel (38) earlier noted that 11% DE was the lower limit for de-esterification of pectin by PE.

Stability of PE Isozymes. The purified forms of PE lost only 15% of their activities during 2 years at 4°C (40°F) with 0.1M NaCl in phosphate buffer (pH 7.5) (8). At 30°C (86°F) PEI was stable for 24 hr at pH 4 and 7, but PEII lost all activity within 6 hr at pH 4. Tested at 30°C (86°F) in single-strength juice reconstituted from concentrate, activity of PEI and crude orange PE declined but HM-PE retained its activity over the 15 days storage.

Versteeg (8) tested the heat stability of the purified PEs in orange juice, pH 4.0, and found that PEII was the least stable, being completely inactivated at 55°C (131°F); PEI was inactivated at 65°C (149°F), and HM-PE at 85°C (185°F). Heat inactivation curves of a crude preparation of orange PE in orange juice indicated that about 5% of the total PE activity was due to HM-PE, 60% to PEI and 30% to PEII.

Cloud Stability and PE Activity. The three forms of PE were tested for destabilization of orange juice cloud (8). Only PEI and HM-PE were active at 5°C (41°F) and 30°C (86°F). PEI was much less active than HM-PE at 5°C (41°F). Versteeg (8) concluded that HM-PE activity was responsible for destabilizing orange juice during storage and that cloud stabilization required heating juice to 90°C (194°F) because HM-PE retained activity in juice heated to less than that temperature. The heat stability of the active HM-PE also accounted for the observations on irregular heat inactivation and cloud stability patterns (43, 44, 45, 46). Bissett et al. (45) reported that heating orange juice to 82°C (180°F) inactivated 94 to 95% of the PE activity but did not greatly improve cloud stability of single-strength or concentrated orange juice. A similar report by Atkins et al. (46) showed that 90% inactivation of PE activity of grapefruit juice heated to 85°C (185°F) did not prevent gelation and clarification in the concentrate.

Identification and characterization of HM-PE as the heat tolerant enzyme responsible for clarifying under-pasteurized citrus juices represent a major breakthrough in understanding the relationship between PE and juice quality. Progress in establishing the role of HM-PE *in vivo*, its origin and relationship to PEI and PEII could lead to procedures to control HM-PE formation during fruit development and maturation. Knowing the distribution of this active form of PE in the structurally defined fruit parts could assist the fruit juice technologist to adopt processing conditions to minimize HM-PE presence in the product.

Limonin D-Ring Lactonase (EC 3.1.1.36)

The need to utilize packinghouse rejects of substandard-quality Navel oranges resulted in investigations on the cause and prevention of bitterness in pasteurized Navel orange juice. Juice from the Navel orange was not bitter when freshly expressed but became bitter on standing (47). Higby (47) isolated several bitter substances from Valencia and Navel orange pulps, and purified and characterized one of them as limonin, a substance previously isolated from seeds of several varieties of citrus (48). Higby suggested that the precursor of limonin is limonoic acid and that it is stable in the intact fruit but is lactonized at the pH of juice. Emerson (49, 50) compared the rate at which bitterness developed in early-season Navel oranges with the rate at which limonin formed from limonoic acid in Valencia orange juice and in water at the pH of the juice and concluded that the precursor in Navel oranges was probably one of the lactone acids rather than the diacid. He also suggested that if the diacid were the precursor of limonin, the reaction would probably be enzyme catalyzed because acid catalysis of the diacid was too slow.

Maier and Beverly (51) used high voltage electrophoresis to separate limonin, limonoic acid and limonoate monolactone in extracts from early-season and later-season Navel oranges and Marsh grapefruit. They were able to show that limonoate monolactone was the primary limonoid in the endocarp and albedo tissues of the early-season fruit and that free limonin and limonoic acid were not natural constituents of healthy intact navel oranges or grapefruit. They also showed that the monolactone was not present in later-season fruit. Maier and Margileth (52) identified the monolactone as limonoic acid A-ring lactone after separating the A- and D-ring lactones by paper electrophoresis and TLC. They also obtained indications of an enzyme in carpellary membrane tissue that converted limonoic acid A-ring lactone to limonin. The enzyme was isolated and purified from peeled seeds (53) and the reaction shown to be specific for the D-ring lactone group. The enzyme catalyzed the lactonization reaction at pH 6 and the hydrolytic reaction at pH 8.0. The enzyme was purified about 200-fold and shown to be essentially free from contaminating proteins by disc electrophoresis (53).

Hasegawa (54) reported on properties of the lactonase. The enzyme purified from grapefruit seeds hydrolyzed limonoids that have the D-ring intact but differ from limonin in the vicinity of the A and A'-rings, namely, obacunone, nomilin and ichangin. Limonin D-ring lactonase was shown to be markedly heat resistant, retaining about 30% of its activity after 5 min at 100°C (54).

Citrus leaves were shown to be the site of limonoic acid A-ring lactone biosynthesis in citrus (55). The lactone accumulated to the level of 2000 ppm in very small leaves but as the leaf grew, the lactone content declined. The lactone content of the fruit increased as the level in the leaves declined. Hasegawa and Hoagland (55) also showed that limonoic acid A-ring lactone was not synthesized in the fruit but in the leaves. The radioactive labeled lactone was isolated from a fruit adjacent to a leaf actively synthesizing it from labeled acetate, indicating that the lactone was synthesized in the leaves and transported to the fruit (55).

Although limonoic acid A-ring lactone has been shown to be a substrate of a lactonase that catalyzes the formation of limonin in citrus fruit, several other enzymes in citrus can also use the A-ring lactone as substrate, however, the products are not bitter products. These enzymes and their reactions are reviewed in the next section.

Enzymes to Degrade Limonin Precursor

Hasegawa et al. (56) detected the enzymic conversion of 19-deoxylimonoic acid 3-methyl¹⁴C ester to the 17-dehydro derivative by albedo tissue slices of Navel oranges. They isolated the product and identified it by TLC as the reaction product formed when the substrate was dehydrogenated by limonoate dehydrogenase (EC 1.1.1)

isolated from cell-free extracts of Arthrobacter globiformis (57). They also showed that the 17-dehydro derivative isolated from the reaction mixture with the albedo tissue could be converted back to the substrate, 19-deoxy-limonoid acid 3-methyl ¹⁴C ester by bacterial limonoate dehydrogenase (56). The citrus and bacterial limonoate dehydrogenases both required NAD for the dehydrogenase reaction (56, 57). Although the citrus enzyme was not isolated, identification of the product of the reaction as the 17-dehydro derivative and isolation of 17-dehydrolimonoid A-ring lactone from Navel orange juice and Navel orange peel (58) indicated that one of the limonoid metabolic pathways in citrus was the dehydrogenation of carbon 17 in the lactone.

The 17-dehydrolimonoid A-ring lactone is not bitter, so the enzymic debittering of fresh Navel orange juice with limonoate dehydrogenase from A. globiformis was suggested (57) and then demonstrated (59). A patent was issued for using limonoate dehydrogenase to debitter citrus juices (60). The pH optimum for the bacterial enzyme activity was much higher (pH 9.5) than juice pH (3.5-4.5), so that the rate of debittering was very slow (59) in juice not adjusted to higher pH. Limonoate dehydrogenase isolated from a *Pseudomonas* sp. had a lower pH optimum (pH 8.0) and showed considerable activity even at pH 4 (61). The *Pseudomonas* enzyme used NADP twice as effectively as NAD, and its activity was stimulated by ZnCl₂. Compared to the A. globiformis dehydrogenase, the *Pseudomonas* enzyme was much more stable at pH 3.5 (62). As little as 200 units of the enzyme decreased the limonin precursor level in one liter of fresh orange juice to 4 ppm from 21 ppm in 2 hr at 24°C (75°F). Prospects look good for use of the *Pseudomonas* dehydrogenase to debitter orange juice commercially (62).

Recently, a nonspecific enzyme capable of degrading the precursor was extracted from Navel orange albedo (63). Nicol and Chandler (63) used a proximate assay for the degrading enzyme; that is, the substrate was not identified except as a limonin precursor. The crude extracts from albedo contained degradation activity that was concentrated by 40 to 60% saturation with (NH₄)₂SO₄. Instability of the enzyme prevented further purification.

Other Enzymes in Citrus Juices

Pectinesterase and limonin D-ring lactonase are the only enzymes known to catalyze reactions that adversely affect the quality of citrus juices. Bruemmer et al. (64) listed other enzymes that have been detected in citrus juices and described some of the reactions that can occur in the juices. None of the reactions appear to noticeably affect the quality of commercial juices. Freshly extracted citrus juices contain esterase (EC 3.1.1.1) (65, 66) and phosphatase (EC 3.1.32) (66, 67) activities. Native substrates in orange juice for peroxidase

(EC 1.11.1.7) (68) and diphenol oxidase (EC 1.10.3.1) (69) have been identified. The potential role of pyruvic decarboxylase (EC 4.1.1.1) catalyzed reaction as a source of acetaldehyde and other aldehydes in juice was discussed (70). Raymond et al. (71) isolated the decarboxylase from orange juice sections and demonstrated that only 10 to 15% of the enzyme was in an active form. Since the purified enzyme was only active with pyruvic acid and 2-ketobutyric acid of the series of 2-ketoacids examined, they (71) concluded that the direct contribution of orange pyruvic decarboxylase to the orange volatile profile was limited to acetaldehyde and possibly propionaldehyde.

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Importance of Selected Volatile Components to Natural Orange, Grapefruit, Tangerine, and Mandarin Flavors

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During the past two decades, researchers have gained much information on compounds responsible for the flavor of the major citrus cultivars, orange, grapefruit, mandarin (tangerine), lemon, and lime. Widespread use of gas chromatography has enabled researchers to identify and quantitate many of the flavor constituents in these citrus cultivars (1, 2, 3). In a limited way, we are able to describe the mixture of components necessary to create the unique flavor of each of the major citrus fruits. However, the contributions to citrus flavor of many of the volatile and nonvolatile citrus components that have been identified are undetermined or only partly characterized. The interrelationship of citrus components and their effect on citrus flavor, particularly orange, is widely known, but few definitive studies have been carried out. Several compounds have been implicated as important to the flavor of certain types of citrus, especially mandarin, but evidence supporting these relationships is not definitive.

In this chapter, we present some specific evidence on certain components important to citrus flavor. The interrelationship of certain volatile components to orange flavor is described and the flavor of grapefruit and the importance of specific compounds to the flavor of mandarin and tangerine are related to recent taste panel studies at our laboratory.

Components Important to Natural Orange Flavor

Orange flavor is one of the most popular and generally accepted fruit flavors; it is widely used in flavoring beverages, candies, and other foods in which fruit flavor notes are desirable. Early research workers felt that the unique flavor of orange could be ascribed to one or a few components of the juice and oil, such as specific aldehydes (4), but extensive analytical studies during the past 20 years have led to the conclusion that a mixture of several compounds in the proper proportions is necessary for a good orange flavor (1).

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In tests to better define this mixture of components (and their proper proportions) necessary for good orange flavor, volatile components believed from prior analytical studies to be important to orange flavor were examined (5). Individual taste and aroma thresholds in water were determined on the compounds selected. Then, the influence of nonvolatile juice constituents on the taste threshold of certain of the volatile components was studied. Finally, selected individual compounds and mixtures containing from two to six components were evaluated in a bland juice medium for their contribution to orange flavor.

Taste and Aroma Thresholds in Water of Individual Orange Juice Components

Table I lists the aroma and taste thresholds in water for 29 orange juice components believed to contribute to orange flavor (6, 7) and the estimated concentrations in orange juice for many of these compounds based on the best data currently available (8-15). For some components, no quantitative data is available for even an estimation of their concentrations in orange juice.

Several difficulties are encountered in trying to estimate quantities of individual components in orange juice. If distillation, extraction with an organic solvent, and derivative formation are used to separate individual components quantitatively, as in the early work of Kirchner and Miller (6), quantitative values will be low because of inevitable losses. Juice free from peel oil (9) will have abnormally low levels of juice components that are also present in the peel oil, because most commercial juices contain 0.015-0.025% oil. Reasonably accurate estimates for most commercial juices prepared from frozen concentrated orange juice can be calculated with quantitative values in peel oil if the oil level is within the above range (e.g., 0.0175% oil). However, single-strength orange juice processed directly from the extractors contains about 0.005% juice oil (16), and thus its oil content would consist of about 75% peel oil and 25% juice oil. Most of the estimates in Table I are calculated on the assumption that all oil in the juice is peel oil. Few accurate quantitative values exist on individual constituents of juice oil because of the difficulty in obtaining a sample uncontaminated with peel oil. Thus, the estimates involving oil constituents must be based on peel oil values only.

Statistical analysis of the aroma and taste threshold values in Table I showed that within 95% confidence limits there was no difference between aroma and taste threshold values for all compounds but four. Octanal and citral had aroma threshold values significantly higher than the corresponding taste threshold values. Nonanal and trans-2-hexenal had higher taste threshold than aroma threshold values.

Comparison of the taste threshold with estimated concentration in orange juice (where available) in Table I reveals that in all cases except octyl acetate and α -pinene, the concentration in orange juice exceeds the taste threshold in water for most values reported. Patton and Josephson (17) postulated that components present in a food at above threshold level make a positive contribution to the flavor, while those present at below threshold level make little or no contribution to flavor. This generalization is now considered an oversimplification, for synergistic effects among food constituents have been shown to decrease the threshold level of some compounds, and nonvolatile constituents are known to either increase or decrease the taste threshold of certain volatile and nonvolatile constituents.

In early studies, the flavor of orange was ascribed to the presence of aldehydes, especially octanal and decanal (18). One taste panel judged 1 ppm aqueous solutions of octanal, nonanal, decanal, or dodecanal to have an orange-like flavor and a bitter taste (7). The aldehydes in Table I most likely to contribute significantly to flavor of orange juice are those with concentrations in juice that greatly exceed the individual taste threshold levels. The estimated concentrations of ethanal (acetaldehyde), octanal, and dodecanal in juice are more than 100 times higher than their taste thresholds. Octanal and dodecanal have lower taste thresholds than would be predicted from values for other straight chain aldehydes in the homologous series. This phenomenon has been observed before (19) for aldehydes with chain lengths in multiples of four carbon atoms.

Of the compounds listed in Table I, ethyl butyrate was present in orange juice at the greatest level above its taste threshold value (2200 times its threshold level). Kefford (18) stated that aldehydes and esters were important to orange flavor. Ethyl butyrate is one of the main esters in aqueous orange essence (2). Its low taste threshold relative to its estimated concentration in juice and its definite contribution to "fruity" flavor notes suggest that ethyl butyrate is a major contributor to good orange flavor. The taste threshold for ethyl butyrate listed in Table I (7) is substantially lower than two earlier reported values for this compound of 450 ppb (20) and 150 ppb (21). The earlier studies involved starting at a high concentration and decreasing the concentration until the compound could not be detected so that fatigue could have been a factor in the relatively high values obtained. The value in Table I was determined by a procedure in which fatigue was not a possible factor (7).

Limonene, a terpene hydrocarbon, is the major component of orange oil (3), and it is present in orange juice at a level almost 800 times its taste threshold in water. Limonene possesses a weak, citrus-like aroma but does not by itself impart an orange-like flavor note to a bland orange juice base (5, 6). Limonene and the other terpene hydrocarbons probably make a significant

Table I. Aroma and taste thresholds in water of selected orange juice components [from Ahmed et al. (7)].

Juice component	Thresholds (ppb) ^a		Concn. (ppb) in orange juice
	Aroma	Taste	
<u>Aldehydes</u>			
Ethanal	17.0	22.0	3,000 ^b
Butanal	15.9	5.26	--
Hexanal	9.18	3.66	40 ^b , 260 ^c
Octanal	1.41	0.52	60 ^b , 510 ^d , 950 ^e
Nonanal	2.53	4.25	160 ^a
Decanal	1.97	3.02	14 ^c , 735 ^d , 1,110 ^e
Dodecanal	0.53	1.07	105 ^d , 138 ^a
<i>trans</i> -2-Hexenal	24.2	49.3	--
Citral	85.3	41.4	14 ^f , 45 ^c , 280 ^e , 385 ^{d,e}
Geranial	--	40	260 ^{a,c}
Citronellal	66	35	40 ^b , 175 ^d
Perillaldehyde	30.1	25.3	5 ^c , 35 ^d
β -Sinensal	3.8	3.8	53 ^d
<u>Hydrocarbons</u>			
Limonene	60	210	4100 ^c , 166,000 ^d
Myrcene	36	42	68 ^c , 2200 ^e , 3500 ^d
α -Pinene	9.5	1013.8	22 ^c , 175 ^e , 875 ^d
<i>p</i> -Cymene	11.4	13.3	12 ^c
<u>Esters</u>			
Ethyl butyrate	0.13	0.13	290 ^c
Ethyl propionate	9.9	4.9	--
Methyl butyrate	43	59	--
Nonyl acetate	57	270	--
Octyl acetate	47	210	175 ^g

Alcohols

Octanol	190	54	10 ^c , 210 ^b
Decanol	47	23	100 ^b
Dodecanol	73	66	--
Linalool	5.3	3.8	150 ^c , 525 ^d , 930 ^b
α -Terpineol	280	300	93 ^c , 320 ^b , 525 ^g

Ketones

d-Carvone	2.7	86.0	35 ^b , 175 ^h
1-Penten-3-one	0.9	1.2	--

^aDetermined with a large group of 55-73 untrained panelists.

^bKirchner and Miller (8).

^cSchreier et al. (9), for juice with no peel oil present.

^dEstimated from data of Shaw and Coleman (10), assuming a level of 0.0175% peel oil in juice.

^eEstimated from the data of Lifshitz et al. (11), for Florida orange oil, assuming a level of 0.0175% peel oil in juice.

^fNaves (12).

^gEstimated from data of Ziegler (13), assuming a level of 0.0175% peel oil in juice.

^hStanley et al. (14), using reported percent aldehyde value (15), and assuming 0.0175% peel oil in juice.

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contribution to orange juice flavor, perhaps as carriers for the oxygenated constituents in cold-pressed oils (22), for concentrated (folded) oils in which much of the terpene hydrocarbons have been removed by distillation have somewhat different characteristics from the corresponding cold-pressed oils. Concentrated oils are used widely in baked goods because they retain their flavors well in the finished products. They are also used in the preparation of certain foods because of their increased solubility due to the decreased amounts of hydrocarbons present.

Effect of Nonvolatile Juice Components on Individual Flavor Thresholds

Interaction of volatile and nonvolatile constituents in foods results in flavor modifications of varying intensities. The effects of 5'-nucleotides on the flavor threshold of octanal (23) and the effects of acid, sugar, and pectin on the flavor threshold of limonene (24) have been studied in orange juice.

Some 5'-nucleotides have been implicated as flavor modifiers, and orange juice is known to contain relatively large quantities of certain 5'-nucleotides, with a total level of about 40 ppm (25, 26). In a study of the influence of six 5'-nucleotides at the 10 ppm level on the taste threshold of octanal in water (Table II), two of the 5'-nucleotides (GMP and ADP) significantly lowered the threshold of octanal (23). GMP is known to enhance flavor in foods, but ADP had been reported to have little or no modifying effect on food flavors (23). Analysis of variance showed that GMP, ADP and GDP enhanced the flavor of octanal in aqueous

Table II. Taste thresholds of octanal determined in aqueous solutions of selected 5'-nucleotides at 10 ppm (23)

5'-Nucleotide	Threshold concn. (ppb) ^a	95% Confidence limits (ppb)
Control (none)	1.38	1.20 - 1.59
ATP (adenosine 5'-triphosphate)	1.22	0.91 - 1.65
GTP (guanosine 5'-triphosphate)	1.21	1.00 - 1.47
AMP (adenosine 5'-monophosphate)	1.20	0.87 - 1.64
GDP (guanosine 5'-diphosphate)	0.99 _b	0.51 - 1.68
GMP (guanosine 5'-monophosphate)	0.86 _b	0.57 - 1.29
ADP (adenosine 5'-diphosphate)	0.85 _b	0.76 - 0.95

^aTwelve screened and trained panelists were employed.

^bSignificantly different from control at 95% level as determined by t-test.

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solution. Thus, this study seems to answer the question raised by Woskow (27) whether 5'-nucleotides enhance the flavor of the food rather than suppress undesirable off-flavors, for no other flavors were present to be suppressed in this study.

Effects of organic acids, sugars, and pectin at the concentrations normally present in orange juice on the flavor threshold of d-limonene in water were also studied by Ahmed et al. (24). Included in that study were flavor effects involving interactions between the nonvolatile constituents of orange juice and effects of the nonvolatile constituents on precision among the taste panel members (Table III). These data show that neither individual nonvolatile components nor combinations of them varied significantly in their effect on the threshold of d-limonene. Because pectin is generally regarded as a thickening agent, its effect on the threshold of d-limonene may be due to textural properties (24).

Table III. Taste thresholds for d-limonene in aqueous solutions containing pectin, acid, and sugar. (24)

Solutions	Thresholds (ppm) ^a	95% Confidence limits (ppm)	Correlation ^b coefficient (r)
Water only	0.21	0.05-0.79	0.82
Acid	0.41	0.17-0.99	0.91
Pectin	0.22	0.07-0.61	0.88
Sugar	0.35	0.12-0.96	0.89
Pectin and acid	0.31	0.08-1.20	0.83
Pectin and sugar	0.23	0.08-0.64	0.89
Acid and sugar	0.38	0.14-1.10	0.88
Pectin, acid, and sugar	0.36	0.13-1.00	0.89

^aTwelve screened and trained panelists were employed.

^bAll correlation coefficients were significant at the 0.01 level.

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When the average effect of each component was evaluated with a t test, some differences were found (Table IV). The taste threshold for limonene in solutions containing acid was significantly higher than that in solutions not containing acid. These results also show a tendency for threshold values to be lower in solutions containing pectin and higher in solutions containing sugar than those without.

Although d-limonene may not be representative of all volatile flavor components in orange juice, these results indicate that acid probably plays an important role in masking the effect of some volatile flavor components. Pangborn (28) reported that in fruit nectar, the greater the acidity, the greater the depressing effect on the intensity of the compound added. The observation that sugar tended to raise the threshold of d-limonene agrees with the reported observation (29) that sweetness beyond that imparted by 15% sugar interferes with flavor perception.

Certain of the nonvolatile compounds affected the ability of panelists to determine the threshold of d-limonene (Table V). Thus, precision appeared high in solutions of water and in the aqueous solutions of sugar alone, but was less in solutions containing pectin and least in solutions containing acid.

Table IV. Average taste threshold of d-limonene in aqueous solutions with and without pectin, acid, and sugar.(24)

Solution	Average thresholds (ppm)
With acid	0.36 ^a
Without acid	0.25 ^a
With sugar	0.33
Without sugar	0.28
With pectin	0.28
Without pectin	0.34

^aSignificantly different at P < 0.05.

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Table V. Percentage of correct responses to blanks in different solutions^a.(24)

Solution	Percentages
Water	90.0
Pectin	72.9
Acid	66.7
Sugar	94.4
Pectin + acid	68.7
Pectin + sugar	76.7
Acid + sugar	73.3
Pectin + acid + sugar	80.0

^aBlank = reference sample presented as unknown.

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Influence of Selected Volatile Components on Flavor of a Bland Orange Juice Drink

In the final phase of their study, Ahmed and coworkers (5, 30) evaluated effects on flavor of a bland orange juice drink by addition of two- to six-component mixtures of some of the volatile flavor compounds used in earlier parts of this project. The bland orange juice drink was concentrated juice from an evaporator (evaporator pumpout) and contained no added peel oil, aqueous essence, cut-back juice, or other flavor fractions and, thus, was

devoid of orange juice flavor except for the sweet and sour notes. This bland orange juice drink, when reconstituted to single strength juice, had the proper balance of sugars, acids, pectin and other nonvolatile components of orange juice without the volatile flavor notes associated with orange juice flavor. Concentrations of compounds added to the drink were those reportedly present in a good quality orange juice (30).

In the first series of extensive tests on single components and up to five component mixtures, juice prepared from good quality commercial frozen concentrated juice was used as a reference and either the bland orange juice drink or mixtures of components in the bland orange juice drink were presented as unknowns to panelists for ranking on a scale of 1 to 7. All components were added to the bland orange juice drink at levels reported by Ahmed et al. (30). Table VI lists the flavor scores for these samples adjusted to good quality juice receiving a rating of 7.0. The samples are listed in order of their increasing flavor ratings for each number of compounds added.

Having a blend of compounds is important in creating an acceptable orange juice flavor (Table VI). Bland orange juice drinks containing most of the single components and some of the two component mixtures received flavor scores lower than that of the bland orange juice drink base; none of the single components afforded a drink with a higher flavor score than the bland orange juice drink. *trans*-2-Hexenal either singly or in combination with other compounds resulted in juices receiving the lowest flavor scores, although the precise negative contribution to flavor was not specified. Three- and four-component mixtures containing limonene, ethyl butyrate, citral, and acetaldehyde received the highest flavor scores of any mixtures evaluated.

The bland orange juice drink received such a high rating hedonically (4.8) that the procedure was modified for the final series of tests in a successful attempt to improve the precision of the sensory data. In the modified test, two reference juices were presented to the panelists in addition to the modified samples. One reference juice contained no volatile components (bland orange juice drink) and the other was a good quality commercial orange juice (Table VII). The sensory score range was expanded to 1 for the bland juice drink reference and 10 for the commercial orange juice. The commercial orange juice was presented periodically as an unknown and scores were adjusted accordingly (Table VII). Commercial orange juice reference sample presented as an unknown received the highest actual sensory score.

Of the mixtures with from three to twelve components, several three- and four-component mixtures received the highest scores, indicating that a highly complex mixture was not necessarily desirable for good orange flavor. Highest scores were obtained for mixtures of citral, ethyl butyrate and *d*-limonene (79%); citral, ethyl butyrate, *d*-limonene, and acetaldehyde (81%); citral, ethyl butyrate, *d*-limonene, and α -pinene (82%); and ethyl butyrate, *d*-limonene, and nonanal (83%).

Table VI. Mean sensory flavor scores of modified bland orange juice drink in comparison to good quality reference juice^a. (30)

Mixtures	Rating		% of maximum
	Actual	Adjusted ^b	
Bland orange juice drink	4.1 ± 0.4	4.8	68
Reference orange juice	6.0 ± 0.1	7.0	100
<u>One component</u>			
<u>trans-2-Hexenal (9000 ppb)</u>	1.7 ± 0.3	2.0	28
<u>(4500 ppb)</u>	1.7 ± 0.3	2.0	28
<u>(3000 ppb)</u>	2.3 ± 0.4	2.7	39
Myrcene	2.8 ± 0.5	3.3	47
d-Limonene	3.5 ± 0.5	4.1	58
Linalool	3.8 ± 0.6	4.4	63
Acetaldehyde	3.9 ± 0.5	4.6	66
Nonanal	3.9 ± 0.4	4.6	66
Citral	4.0 ± 0.4	4.7	67
Citronellal	4.0 ± 0.6	4.7	67
Dodecanal	4.0 ± 0.5	4.7	67
Octanal	4.0 ± 0.3	4.7	67
α-Pinene	4.1 ± 0.6	4.8	68
Ethyl butyrate	4.1 ± 0.8	4.8	68
<u>Two components</u>			
<u>trans-2-Hexenal (3000 ppb) and linalool</u>	1.6 ± 0.6	1.9	27
<u>trans-2-Hexenal (3000 ppb) and acetaldehyde</u>	1.7 ± 0.7	2.0	28
<u>trans-2-Hexenal (3000 ppb) and octanal</u>	1.8 ± 0.6	2.1	30
<u>trans-2-Hexenal (3000 ppb) and ethyl butyrate</u>	1.9 ± 0.5	2.2	31
<u>trans-2-Hexenal (3000 ppb) and limonene</u>	2.2 ± 0.4	2.6	37
<u>Acetaldehyde and citral</u>	2.4 ± 0.6	2.8	40

Limonene and octanal	3.3 ± 0.5	3.8	54
Limonene and α-pinene	3.6 ± 0.6	4.2	60
Acetaldehyde and ethyl butyrate	3.7 ± 0.5	4.3	61
Acetaldehyde and limonene	4.0 ± 0.4	4.7	67
Ethyl butyrate and α-pinene	4.1 ± 0.5	4.8	68
Citral and dodecanal	4.1 ± 0.6	4.8	68
Citral and α-pinene	4.1 ± 0.6	4.8	68
Acetaldehyde and octanal	4.2 ± 0.4	4.9	70
Citral and octanal	4.2 ± 0.4	4.9	70
Acetaldehyde and linalool	4.3 ± 0.4	5.0	71
Acetaldehyde and α-pinene	4.4 ± 0.5	5.1	73
Linalool and octanal	4.4 ± 0.5	5.1	73
Acetaldehyde and dodecanal	4.5 ± 0.5	5.2	74
Ethyl butyrate and linalool	4.5 ± 0.4	5.2	74
Citral and ethyl butyrate	4.6 ± 0.4	5.4	77
<u>Three components</u>			
Limonene, α-pinene, octanal	4.0 ± 0.4	4.7	67
Limonene, acetaldehyde, octanal	4.2 ± 0.3	4.9	70
Limonene, ethyl butyrate, octanal	4.5 ± 0.4	5.2	74
Limonene, citral, acetaldehyde	5.1 ± 0.4	6.0	86
Limonene, citral, ethyl butyrate	5.2 ± 0.3	6.1	87
<u>Four components</u>			
α-Pinene, citral, ethyl butyrate, acetaldehyde	4.6 ± 0.5	5.4	77
Limonene, acetaldehyde, ethyl butyrate, octanal	4.6 ± 0.4	5.4	77
Limonene, citral, ethyl butyrate, acetaldehyde	5.0 ± 0.3	5.8	83
<u>Five components</u>			
Limonene, citral, ethyl butyrate, acetaldehyde, α-pinene	4.3 ± 0.4	5.0	71

^aSensory scores: 7 = similar to, and 1 = dissimilar to reference juice (mean ± S.D.).

^bAdjustment factor 1.1667.

Table VII. Mean sensory flavor scores of modified bland orange juice drink in comparison to two references representing extremes of flavor quality^a, (30)

Mixtures	Rating		% of maximum
	Actual	Adjusted ^b	
Bland orange juice drink	1.5 ± 0.5	2.0	20
Commercial orange juice	7.6 ± 0.1	10.0	100
<u>Three components</u>			
d-Limonene, decanal, α-pinene	3.1 ± 0.4	4.1	41
d-Limonene, decanal, linalool	3.4 ± 0.4	4.5	45
Citral, decanal, d-limonene	3.5 ± 0.5	4.6	46
Ethyl butyrate, d-limonene, decanal	3.8 ± 0.5	5.0	50
Acetaldehyde, citral, octanal	3.9 ± 0.4	5.2	52
Acetaldehyde, citral, α-pinene	4.0 ± 0.3	5.3	53
Acetaldehyde, citral, decanal	4.0 ± 0.4	5.3	53
Acetaldehyde, decanal, d-limonene	4.2 ± 0.4	5.5	55
Acetaldehyde, citronellal, α-pinene	4.4 ± 0.4	5.8	58
Acetaldehyde, citral, citronellal	4.6 ± 0.4	6.1	61
Citral, citronellal, octanal	4.6 ± 0.4	6.1	61
Acetaldehyde, d-limonene, linalool	5.2 ± 0.3	6.2	62
d-Limonene, citronellal, linalool	4.7 ± 0.4	6.2	62
Acetaldehyde, citronellal, d-limonene	4.8 ± 0.4	6.3	63
Citral, d-limonene, α-pinene	4.8 ± 0.4	6.3	63
Citral, d-limonene, citronellal	4.8 ± 0.4	6.3	63
Citral, ethyl butyrate, octanal	4.8 ± 0.3	6.3	63
Citral, octanal, α-pinene	4.8 ± 0.4	6.3	63
Acetaldehyde, d-limonene, nonanal	4.9 ± 0.4	6.5	65
Citral, linalool, octanal	4.9 ± 0.4	6.5	65
Acetaldehyde, citral, d-limonene	5.0 ± 0.4	6.6	66
Acetaldehyde, citral, linalool	5.1 ± 0.4	6.7	67

Acetaldehyde, d-limonene, octanal	5.1 ± 0.3	6.7	67
Citral, d-limonene, nonanal	5.1 ± 0.3	6.7	67
Ethyl butyrate, citronellal, d-limonene	5.1 ± 0.4	6.7	67
d-Limonene, linalool, α-pinene	5.1 ± 0.5	6.7	67
d-Limonene, nonanal, α-pinene	5.1 ± 0.5	6.7	67
Citral, d-limonene, octanal	5.3 ± 0.3	7.0	70
Citral, decanal, linalool	5.4 ± 0.4	7.1	71
Acetaldehyde, d-limonene, α-pinene	5.5 ± 0.3	7.3	73
Citral, citronellal, ethyl butyrate	5.5 ± 0.4	7.3	73
Citral, ethyl butyrate, α-pinene	5.5 ± 0.3	7.3	73
Ethyl butyrate, d-limonene, octanal	5.5 ± 0.3	7.3	73
Acetaldehyde, citral, ethyl butyrate	5.7 ± 0.3	7.5	75
Citral, d-limonene, linalool	5.7 ± 0.4	7.5	75
Citral, ethyl butyrate, linalool	5.8 ± 0.3	7.7	77
Ethyl butyrate, d-limonene, linalool	5.8 ± 0.4	7.7	77
Citral, ethyl butyrate, d-limonene	6.0 ± 0.2	7.9	79
Ethyl butyrate, d-limonene, nonanal	6.3 ± 0.5	8.3	83
<u>Four components</u>			
Acetaldehyde, citral, ethyl butyrate, d-limonene	4.3 ± 0.4	5.7	57
Acetaldehyde citral, d-limonene, α-pinene	4.7 ± 0.4	6.2	62
d-Limonene, citronellal, linalool, α-pinene	4.9 ± 0.5	6.5	65
Citral, ethyl butyrate, d-limonene, α-pinene	5.0 ± 0.3	6.6	66
d-Limonene, citronellal, linalool, octanal	5.1 ± 0.5	6.7	67
Acetaldehyde, ethyl butyrate, d-limonene, octanal	5.4 ± 0.4	7.1	71
Citral, ethyl butyrate, d-limonene, linalool	5.7 ± 0.4	7.5	75
Citral, citronellal, ethyl butyrate, d-limonene	5.8 ± 0.4	7.7	77
Acetaldehyde, citral, ethyl butyrate, d-limonene	6.1 ± 0.2	8.1	81
Citral, ethyl butyrate, d-limonene, octanal	6.3 ± 0.3	8.2	82
<u>Five components</u>			
Citral, ethyl butyrate, d-limonene, octanal, α-pinene	5.0 ± 0.4	6.6	66
Acetaldehyde, citronellal, d-limonene, linalool, α-pinene	5.3 ± 0.4	7.0	70

Acetaldehyde, ethyl butyrate, citral, d-limonene, octanal	6.1 ± 0.2	8.1	81
<u>Six-twelve components</u>			
Acetaldehyde, citral, citronellal, decanal, ethyl butyrate, d-limonene, linalool, myrcene, nonanal, octanal, α -pinene, trans-2-hexenal	1.1 ± 0.1	1.5	15
Acetaldehyde, citral, citronellal, decanal, ethyl butyrate, d-limonene, linalool, octanal, α -pinene	3.4 ± 0.4	4.5	45
Acetaldehyde, citral, citronellal, ethyl butyrate, d-limonene, octanal	4.2 ± 0.4	5.5	55
Acetaldehyde, citral, citronellal, ethyl butyrate, d-limonene, linalool, octanal, α -pinene	5.7 ± 0.3	7.5	75

^aSensory scores: 1 similar to pumpout juice, 10 similar to reference juice. Actual data presented as mean \pm standard deviation. All mixtures were prepared by adding components to bland orange juice drink at levels reported by Ahmed et al. (30).

^bBased on reference orange juice score as 100% of maximum.

These studies suggest that mixtures of d-limonene, ethyl butyrate, and certain aldehydes seem to be best at providing good orange flavor when mixtures of only a few flavor components are used. Mixtures of more than four components seemed to have less desirable flavoring qualities than simpler three- and four-component mixtures. Precise quantitative values for individual flavor components in citrus juices are still lacking (3). Perhaps in the mixtures containing five or more flavor components, where interactions between components are accentuated, a blend of components more nearly identical to that naturally occurring in orange juice is critical to avoid creation of undesirable flavor effects due to these interactions.

Components Important to Natural Grapefruit Flavor

Grapefruit flavor has become increasingly popular in recent years, partly because fresh grapefruit are now being distributed worldwide in increasing quantities, and partly because synthetic nootkatone, the primary flavor impact component of grapefruit, is available for use in synthetic grapefruit-flavored beverages and other foods. Since the discovery and synthesis of nootkatone (31, 32), food flavorists have discussed its relative importance in grapefruit flavor, and whether it is even necessary for good grapefruit flavor (33).

Nootkatone. The taste threshold of nootkatone was first reported to be 20-40 ppm in sucrose solution, and the odor was detectable at less than 10 ppm (31). MacLeod and Buigues estimated the level of nootkatone in juices free from peel oil at about the taste threshold level found in sucrose solution (31). Their data of 77 mg of crude nootkatone isolated from 1 gal of peel-oil-free juice and rag represent a level of about 20 ppm nootkatone in the juice.

Taste thresholds of 1 ppm nootkatone in water and 5-6 ppm in grapefruit juice were determined by Berry et al. (34), who stated that grapefruit juice containing 0.005% oil contains an average level of less than 0.5 ppm nootkatone, which is considerably lower than the threshold level in juice. They considered only the nootkatone present in the oil, and any nootkatone present in the oil-free juice would have added to this value.

Some reported odor threshold values for nootkatone were considerably lower than the taste threshold values. Odor thresholds of 0.8 ppm in water and 30 ppm in air were reported for (+)-nootkatone, the enantiomer of this sesquiterpene ketone present in grapefruit (35). An odor threshold of 0.15 ppm in water was reported for crystalline nootkatone isolated from grapefruit oil (36). In that study, mother liquor from crystallization of nootkatone was 30 times more potent (odor threshold of mother liquor 5 ppb) than nootkatone alone and the panel felt that the aroma of the mother liquor more closely resembled grapefruit aroma

than did that of crystalline nootkatone. When synthetic nootkatone is used to flavor grapefruit-flavored products, the technical grade, which contains more impurities, is generally preferred over the pure grade (37). Thus, it is clear that other grapefruit oil components, probably similar to nootkatone in chemical structure, contribute to the flavor and aroma of grapefruit oil.

We carried out a recent study to better define the role of nootkatone in the aroma and flavor of cold-pressed grapefruit oil (33). We obtained good quality commercial cold-pressed grapefruit oils with relatively high and low levels of nootkatone and compared their aromas with those of limonene (the major component of grapefruit oil) and nootkatone in limonene at the level corresponding to that in the high-nootkatone grapefruit oil sample. Then we added these oils to single-strength juice from commercially prepared frozen concentrated grapefruit juice that contained no added peel oil or flavor fractions (other than the oil we added) and compared the taste of the resulting juices (Table VIII). In all cases an experienced 12-member panel was used.

Table VIII. Effect of nootkatone content on aroma and taste of grapefruit oil and juice.

Oil samples compared ^a	Confidence limit (%)	
	Aroma panel ^b with oils	Taste panel ^c with juices
Limonene vs. high-nootkatone oil	-	99.9
Limonene vs. nootkatone in limonene	99	N.S.
Nootkatone in limonene vs. high-nootkatone oil	99	95
Low-nootkatone oil vs. high-nootkatone oil	N.S.	N.S.
Nootkatone + low-nootkatone oil vs. high-nootkatone oil	95	N.S.

^aHigh-nootkatone oil, nootkatone in limonene, and nootkatone + low-nootkatone oil all contain 0.83% nootkatone; low-nootkatone oil contains 0.02% nootkatone.

^bPaired comparison difference test.

^cTriangle comparison difference test.

The taste panel could readily distinguish a sample of juice containing high-nootkatone (0.83%) grapefruit oil from a sample of juice containing limonene at the same effective oil level, thus showing that flavor of the grapefruit juice did not mask the effect of added grapefruit oil. In aroma tests with limonene samples, the panel distinguished 0.83% nootkatone in limonene from limonene and 0.83% nootkatone in limonene from high-nootkatone oil

at the 99% confidence level. However, the taste of juice containing these samples was less affected. The panel was unable to distinguish juice containing only added limonene from juice containing 0.83% nootkatone in limonene, but it could distinguish juice containing high-nootkatone oil from that containing nootkatone in limonene at the 95% confidence level. These results indicate the importance of constituents other than nootkatone to the flavor of grapefruit juice containing added cold-pressed oil.

Comparison of low- and high-nootkatone oils generally showed insignificant differences in aroma or taste. Low-nootkatone oil was not significantly different from high-nootkatone oil either by aroma of the neat oils or by taste imparted to single-strength grapefruit juice. When crystalline nootkatone was added to low-nootkatone oil to raise the level of this compound to that in the high-nootkatone oil, the aromas of the two oils were significantly different, but juices flavored with the two oils could not be distinguished by taste. Apparently, the added nootkatone modified the flavor of the low-nootkatone oil, perhaps through synergistic effects with other oil components. However, the nootkatone level in the oil is insufficient to cause dramatic changes in the taste of grapefruit juice flavored with these oils.

One critical factor in the effect of cold-pressed grapefruit oil containing nootkatone on the flavor of grapefruit juice is the level of nootkatone present in the juice prior to addition of the oil. We estimated the level of nootkatone in our grapefruit juice sample to be 7 ppm by thin-layer chromatography (33, 38). This is within the range of 6-7 ppm nootkatone reported (34) for optimum flavor. Thus, addition of even high-nootkatone oil adds only about 0.8 ppm to this value, which is already above the threshold level. It is perhaps not surprising that the taste panel had difficulty distinguishing high-nootkatone oil from low-nootkatone oil in single-strength grapefruit juice. This situation is comparable to that found for commercial samples of grapefruit juice flavored with added cold-pressed oil, since both frozen concentrated grapefruit juice and single-strength juice would be expected to contain nootkatone prior to addition of cold-pressed oil (31). Nootkatone is one of the less-volatile oil components so that frozen concentrated and deoiled single-strength juice might be expected to have higher nootkatone levels than the oil content as determined by standard methods might indicate.

Aldehydes and Esters. Aldehydes have long been considered important to the flavor of grapefruit and other cold-pressed oils from citrus. The total level of aldehydes in Florida grapefruit oil reaches a maximum in fruit harvested during mid-season (39). Kesterson et al. (39) stated that optimum quality grapefruit oils contain the highest level of total aldehydes (about 1.8%) and a moderately high level of nootkatone (0.5-0.7%). Thus, the aldehydes are clearly important to grapefruit oil quality. Esters have also been implicated as important contributors to flavor of

grapefruit oil (40). Quantities of several of these esters have recently been determined in grapefruit oil (41), but their precise contribution to grapefruit flavor has not been determined.

Although nootkatone is a flavor impact compound in grapefruit, it does not by itself provide the full-bodied flavor provided by good quality cold-pressed grapefruit oil. The proper blend of nootkatone, aldehydes, other as yet unidentified components, and perhaps esters is necessary for full-bodied grapefruit flavor.

Components Important to Mandarin Flavor and Aroma

The flavor of most citrus cultivars is complex, and compounding citrus flavors requires the blending of several components in specific proportions to obtain the unique flavor of each citrus cultivar (3). Studies on Sicilian mandarin oil suggest the distinct flavor and aroma of mandarins is mainly due to 2 compounds, thymol and methyl-N-methyl anthranilate (dimethyl anthranilate) but no evidence to support this claim has been presented (42). Thymol has been identified in Dancy tangerine peel oil, and both thymol and dimethyl anthranilate have been identified in Sicilian mandarin oil (3). The reported quantities of thymol in mandarin oil varied from 0.04-0.2% of the oil, whereas only one value (0.9%) was reported for dimethyl anthranilate.

In a recent study (44), we determined the quantities of thymol and dimethyl anthranilate in Argentine (Sicilian) mandarin oil and in cold-pressed tangerine oil. We assessed the flavor and aroma effects of these two compounds on cold-pressed oils from mandarins and tangerines and determined the taste threshold of dimethyl anthranilate in water, and of thymol and dimethyl anthranilate in single-strength tangerine juice. The taste threshold of thymol in water was reported earlier (43). We prepared oil samples for aroma and taste evaluation by adding mixtures of thymol and dimethyl anthranilate or thymol, dimethyl anthranilate, α -pinene and γ -terpinene, to either cold-pressed tangerine oil or d-limonene (the major component of both tangerine and mandarin oils) at the levels found in mandarin oil. α -Pinene, γ -terpinene, thymol, and dimethyl anthranilate were quantitated in tangerine and mandarin oil by gas chromatography on a methyl silicone, fused silica, glass capillary column (Table IX) (44). Aroma samples were evaluated by a paired comparison difference test using a trained twelve-member aroma panel. Then, the oil samples were added to single-strength tangerine juice and evaluated by a triangle difference test with the same twelve-trained panel members (Table X). We determined taste thresholds in water by adding an appropriate amount of test material in ethanol to water, and in single-strength juice by adding dimethyl anthranilate or a solution of thymol in ethanol to the concentrated tangerine juice and diluting to single-strength juice with water (Table XI).

The aroma and taste panel results (Table X) point to some significant conclusions about the effect of thymol and dimethyl anthranilate on the aroma and taste of mandarin oil. The aroma panels readily detected a difference in samples of tangerine oil containing added thymol and dimethyl anthranilate that were compared to mandarin oil but did not detect a difference as easily when thymol and dimethyl anthranilate in limonene were compared to mandarin or tangerine oils, or when thymol and dimethyl anthranilate in tangerine oil were compared to tangerine oil. Taste panel results show that panel members readily detected a difference in all samples tested. These results suggest that a combination of thymol and dimethyl anthranilate is not as important to the flavor and aroma of mandarin oil as originally proposed. They also show that tangerine oil contains other constituents that modify the flavor and aroma of the oil even when thymol and dimethyl anthranilate are adjusted to the same levels found in mandarin oil. In one aroma test, however, when α -pinene and γ -terpinene were added to tangerine oil along with thymol and dimethyl anthranilate, the panel could not distinguish the compounded oil from mandarin oil. These results indicate the importance of monoterpene hydrocarbons to the aroma of mandarin oil. The taste panel readily detected a taste difference in this same sample of oil in single-strength juice. Thus, it is apparent that other citrus oil components modify the taste of mandarin oil.

Table IX. Quantities of important flavor components in mandarin and tangerine oils.

Component	Weight percent	
	Tangerine oil	Mandarin oil
Thymol	0.022	0.182
Dimethyl anthranilate	0.072	0.652
γ -Terpinene	1.74	14.0
α -Pinene	0.17	1.8

The taste thresholds for dimethyl anthranilate and thymol (Table XI) indicate the effect of these two components on the aroma of mandarin oil and the flavor of tangerine juice containing mandarin oil. Dimethyl anthranilate is by far the more potent flavor component, for it is detectable at a level that is one-hundredth that of thymol in water and one-thousandth that of thymol in bland tangerine juice (Table XI). We estimated the levels of thymol and dimethyl anthranilate in tangerine juice flavored with mandarin oil to be 220 ppb of thymol and 782 ppb of dimethyl anthranilate based on a level of 0.012% oil in the juice. The level of dimethyl anthranilate present is about five times its taste threshold in tangerine juice, but the level of thymol is almost one seven-hundredth (1/700) its taste threshold in tangerine juice. Thus, dimethyl anthranilate is much more likely to

Table X. Contribution of thymol, dimethyl anthranilate, β -pinene and γ -terpinene to the aroma and taste of coldpressed tangerine and mandarin oils.

Oil samples compared	Confidence level ^a	
	Aroma panel ^b with oils	Taste panel ^c with juices
Tangerine vs mandarin	99.9	99.9
Tangerine, DMA vs mandarin	99.9	99.9
Tangerine, thymol, DMA vs mandarin	99.9	99.9
Limonene, DMA vs mandarin	99.9	99.9
Limonene, thymol, DMA vs mandarin	95.0	99.9
Tangerine, thymol, DMA, γ -terpinene vs mandarin	95.0	
Tangerine, thymol, DMA, γ -terpinene, β -pinene vs mandarin	NS	99.0
Limonene, thymol, DMA vs tangerine	95.0	99.9
Tangerine, thymol, DMA vs tangerine	95.0	99.0

DMA = dimethyl anthranilate.

NS = not significant at the 95% confidence level.

^aDifference between the two samples evaluated.

^bPaired comparison difference test.

^cTriangle comparison difference test.

influence the flavor of juices containing Argentine mandarin oil than is thymol.

Table XI. Taste threshold values for thymol and dimethyl anthranilate in water and in tangerine juice.

Compound	Threshold (ppb)	r ²
In water		
Thymol	1,700 ^a	ND
Dimethyl anthranilate	20.3	0.98
In tangerine juice		
Thymol	147,000	0.93
Dimethyl anthranilate	157	0.96

^aReported earlier by Moshonas et al. (1972); r² not determined (ND).

Taste panel members felt that dimethyl anthranilate was definitely a flavor impact compound in mandarin oil. These studies suggest however, that a mixture of components including thymol, dimethyl anthranilate, and several monoterpene hydrocarbons in the proper proportions is necessary for acceptable flavor in mandarins.

Total Aldehydes As a Measure of Oil Quality

One of the standards of purity for citrus oils is the total aldehyde content determined by a procedure described in the U. S. Pharmacopoeia (USP) (45). Some buyers or processors of citrus essential oils have been unable to duplicate total aldehyde values on a given oil because of slight variations in carrying out the standard procedure. Specifically, Dennis and Hendrix (46) have found that if the solution of peel oil and hydroxylamine hydrochloride is not stirred (or allowed to stand with occasional shaking) for a full 30 min before titration, a low total aldehyde value may result. Varying this time interval can cause variations of as much as 0.25% in the aldehyde value for a given oil. Samples of pure decanal, octanal, and citral diluted to about 1.5% solutions were quantitatively accounted for, showing that the major aldehydes in citrus oils react completely in the USP procedure. A maximum total aldehyde value is highly desirable when an oil is being sold on the basis of its aldehyde content, as is often the case.

Recent quantitative studies on individual aldehydes in citrus oils involving gas chromatography have suggested that total aldehydes in some oils may be higher than total aldehydes obtained by the USP procedure (3, 41). Although the reasons for this difference are unclear, the calculation of total aldehydes in orange, grapefruit, and tangerine oils as decanal may cause some

error, particularly since octanal is sometimes the major aldehyde in these oils. Another reason this difference may exist is the possibility that gas chromatographic peaks assigned to some individual aldehydes may contain impurities, thus creating abnormally high total aldehyde values. This possibility is less likely when analyses are being carried out on glass capillary gas chromatographic columns where contamination of peaks by trace impurities is minimized.

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Fruit Handling and Decay Control Techniques Affecting Quality

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Information concerning various pre- and postharvest techniques used in the handling and processing of citrus fruits which affect quality has been compiled and summarized within the scope of this chapter. Major cultural and genetic influences on quality are not considered. Quality is influenced by physiological and pathological factors and is measured in both specific and general terms. Quality parameters involved with internal composition are usually defined, but in many instances causes related to off-flavors have not yet been identified. Factors which detract from the external appearance and affect the saleability of citrus fruit definitely have to be considered when discussing quality. These factors associated with the rind include color, softness, and various forms of rind breakdown. Keeping quality is another important aspect, and various handling and decay control techniques influence the occurrence of decay caused by several different fungal pathogens. Quality can be adversely affected by many of the techniques, but several specific methods have been developed to improve quality at harvest and/or maintain it during storage or transit.

Preharvest techniques which influence quality

Lead arsenate sprays. Internal fruit quality can be influenced by spraying trees with lead arsenate at post-bloom (1). Oranges (*Citrus sinensis* (L.) Osbeck) are more affected than grapefruit (*Citrus paradisi* Macf.). The use of lead arsenate to reduce titratable acidity of Florida grapefruit has been a standard procedure for many years. Red grapefruit sprayed with lead arsenate contained significantly less acid than did the unsprayed fruit (2). Acid in fruit sprayed with the highest rate (1.9 g/l) was only half that with the lowest rate (0.5 g/l). In addition to acidity reduction, arsenated grapefruit contained slightly less reducing sugar, significantly more non-reducing

sugar, and more total sugar than nonarsenated fruit. The bioflavonoid content and pH were also significantly higher in the sprayed fruit (3). Applications of lead arsenate to Temple oranges lowered the titratable acid content but not the soluble solids or percentage juice (4). Decay, peel injury, or creasing (a rind malformation) were not influenced but legal maturity was advanced by 15 to 20 days. Florida regulations (5) restrict the use of lead arsenate to grapefruit.

Color. Color of citrus fruit at maturity is a major criterion of consumer appeal. In humid, subtropical climates, citrus fruits will reach maturity with a considerable amount of chlorophyll still remaining in the rind. Therefore, considerable interest has been shown in the area of color enhancement through the use of materials applied to mature fruit before harvest. The final color of most orange cultivars is produced by a decline in chlorophyll pigments and an accumulation of carotenoids. The final color on most lemon (*Citrus limon* L. Burm.), lime (*Citrus aurantifolia* (Christm.) Swing) and grapefruit cultivars is produced by a decline in chlorophyll pigments and little or no net increase in carotenoids (6). Removal of chlorophyll from citrus fruits with preharvest applications of ethephon (2-chloroethylphosphonic acid), which decomposes to produce ethylene (7), has been successfully achieved (8, 9, 10, 11, 12). Concentrations between 50 to 200 mg/l as a preharvest spray 5 to 20 days before harvest to Robinson and Lee tangerines (*Citrus reticulata* Blanco) resulted in partial chlorophyll loss (degreening). Less postharvest degreening was required to achieve acceptable color, and keeping quality of the fruit during storage was improved (10, 11). Concentrations of 200 to 500 mg/l induced varying degrees of fruit loosening and, often, fruit drop of Robinson, Lee, Nova, and Dancy tangerines and Hamlin oranges (10). Leaf abscission also occurred, particularly at the higher concentration. Damage to fruit by "plugging", removal of a portion of the peel at the stem-end at harvest, was reduced by the ethephon sprays. Preharvest applications of ethephon to Robinson tangerines significantly reduced the incidence of anthracnose caused by *Colletotrichum gloeosporioides* (Penz.) Sacc. during storage (13). Control was attributed to physiological changes within the rind associated with carotenoid accumulation which occurred due to the presence of low levels of ethylene. All cultivars of citrus, however, do not respond to ethephon treatment. Preharvest applications to Bearss lemons were relatively ineffective for inducing degreening even though the ethephon was absorbed and ethylene was produced (9). Similar rates of application as a postharvest dip induced degreening, suggesting that a factor such as auxin, gibberellin, or cytokinin from the tree inhibited the response to ethylene. El-Zeftawi and Garrett (8) observed that ethephon markedly reduced acidity of juice from green-colored Valencia oranges. In fruit

excluded from light after ethephon application, flavonoids and polyphenols were increased in juice from green-colored fruit or fruit undergoing regreening. Carotenoids were also increased at the orange-colored and regreened (additional synthesis of chlorophyll) stages, but decreased at the green stage. Ethephon did not cause earlier maturation of Shamouti oranges (14).

Growth regulators. Gibberellic acid (GA), 2,4-dichlorophenoxyacetic acid (2,4-D), and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) are growth regulators evaluated on citrus fruits to improve quality at/or after harvest. In general, significant changes in internal quality have not been observed with these growth regulators (6, 15, 16, 17, 18, 19, 20), their effects being restricted primarily to the rind. The use of GA has been particularly successful on Washington Navel oranges grown in most citrus regions. Applications of GA retard senescence of the rind due to high enzymatic and metabolic activities late in the season (21, 22) which delay loss of chlorophyll and accumulation of carotenoid pigments (6, 15, 16, 17, 23, 24, 25, 26, 27, 28). Applications of 2,4-D or closely related compounds have been successfully combined with GA. The effect of 2,4-D has been principally to reduce fruit drop or increase fruit size due to a thinning effect (6, 15, 17, 28, 29, 30, 31, 32). Applications of GA and 2,4-D have been shown to extend the harvesting season of grapefruit (30, 31, 33, 34), producing a firmer fruit and, in some instances, increasing fruit size (33). Fruit treated with these growth regulators withstood deformity due to handling and packing significantly better than untreated fruit (34). Gibberellic acid and 2,4-D alone and in combination reduced germination of seed in mature grapefruit (30). Darkened blemishes, some discoloration of the rind and increased rind thickness were some of the undesirable effects of GA on grapefruit (33).

Regreening due to the application of GA can be a problem on Valencia oranges and grapefruit (15, 30, 35, 36, 37), and fruit deformity has occasionally been observed with Valencia oranges (35). Postharvest decay of fruit sprayed with growth regulators has been reported to be reduced (15, 16, 17, 23, 31). This has been attributed to fewer injuries being formed in firm fruit receiving growth regulators as well as to growth of the decay organisms being decreased in less senescent peel (16). Anatomical changes associated with rind senescence were retarded by GA sprays (6, 16). Gibberellic acid caused an increase in valencene, an essential oil component (27). Valencia oranges sprayed with 2,4-D and GA were firmer, evolved less ethylene, and showed slower malonic acid accumulation in the juice (20). Gibberellic acid reduced Brix (primarily sugars) and acidity in green-colored Valencia oranges (8). Chlorophenoxyacetic acid (CLPA) and 2,4,5-T applied to mandarins increased juice content and peel moisture. Treated fruit suffered less loss of weight, vitamin C, and sugar during storage (38). Gibberellic acid

applied to lemon trees caused the fruit to remain greener and to develop the yellow lemon color less rapidly in storage (39). The benefits of GA applications to lemons are a more desirable seasonal harvest pattern in relation to market demands, a larger percentage of fruit with a long storage life, and a decrease in the number of small fruit (6). Applications of GA to Clementine mandarins significantly delayed fruit coloring, loss of acid, and an increase in soluble solids (40).

Growth regulators have been successfully used to control physiological rind disorders. Rind staining of navel oranges was reduced with GA applications (41, 42, 43), as was the accumulation of rind exudate on mature fruit (44). Water spot of navel oranges, caused by surface deposits of water entering the rind, can be reduced by GA sprays (45, 46). Rough and thick peel of large fruit sizes of Shamouti oranges was reduced significantly by applying 2,2-dimethylhydrazide (SADH) and 2-chloroethyltrimethylammonium chloride (CCC) (47). GA also reduced puffing and creasing of oranges (23, 37, 48, 49). Granulation in Valencia oranges was reduced with 2,4-D sprays (29). Preharvest sprays of benzyladenine, a cytokinin, and 2,4-D tended to decrease susceptibility of grapefruit to chilling injury, a cold storage disorder of the rind (50). Additional information on the effect of growth regulators on citrus has been compiled by Coggins and Hield (6).

Aging, a wrinkling of the peel at the stem-end of Valencia oranges caused from loss of moisture, was reduced by applying Pinolene (poly-1-p-menthen-8,9-diyl), an antitranspirant plastic, 1-2 months before harvest. At both 1 and 3%, Pinolene reduced stem-end aging and moisture loss of Valencia oranges during storage (51). Soluble solids and acidity were reduced, but internal quality was still acceptable (52).

Handling techniques at harvest which influence quality

Harvest methods. Citrus fruits are harvested almost entirely by hand. Initially, citrus fruits were clipped when harvested because the stem does not separate easily from the fruit at picking. However, as this procedure was slow and a certain amount of clipper injury occurred, harvesting by pulling became the predominant method (53). Pulling or snapping, a breaking-by-bending process, causes the stem to break close to the fruit. In many instances, all or portions of the button (calyx and disk) may be removed from the fruit. The button harbors fungi which may later cause decay during storage and thus reduce keeping quality. A comparison of decay in pulled versus clipped fruit showed that stem-end rot, caused by *Diplodia natalensis* P. Evans and *Phomopsis citri* Fawc., was reduced by pulling (53, 54, 55). Levels of decay caused by *Alternaria citri* Ell. and Pierce, however, were increased (55). Spot picking, principally on the basis of color, can be used to

aid in the harvest of high quality fruit. Sites and Reitz (56, 57, 58) showed that the best colored fruit contained the highest soluble solids and vitamin C content, and usually had a higher soluble solids/titratable acid ratio. Spot picking for juice content had no practical application (58).

Unnecessary rough handling, no matter whether fruit are harvested by clipping or pulling, causes injuries to the rind which are readily infected by decay fungi. The simple practice of careful handling during harvesting has an indubitably significant influence upon quality. It has been shown in numerous studies that rough handling significantly reduces keeping quality of citrus fruits (59, 60, 61, 62) and quality decreases with increased severity of the injuries (61). Gently handled grapefruit held under humid, shaded conditions until packing were more resistant to deformation than fruit roughly-handled and exposed to the sun. Susceptibility to permanent deformation increased with advancing maturity (64).

Physiological disorders. Physiological disorders are also influenced by handling at harvest. Blossom-end clearing is a symptom of bruising which is common on fully mature thin-skinned, seedless grapefruit that have been subjected to rough handling (63). This disorder is usually visible within 24 hours after bruising and is manifested by water soaking of the blossom-end caused by exudation of juice from broken vesicles within the pulp. The disorder is more prevalent in rough handled mature fruit (62). Oleocellosis or oil spotting occurs when fruit are injured during handling causing a release of oil from damaged glands. The oil is toxic to surrounding healthy cells of the rind causing a collapse and discoloration of the area (65). Turgid fruit are more subject to the disorder (42, 66, 67, 68, 69, 70) and harvesting should preferably be delayed until the rate of water loss from the tree exceeds the rate of water uptake. Fruit harvested while wet or damp from rain, overhead irrigation, dew or fog are very susceptible to oleocellosis. Damaged areas collapse and darken, causing the oil glands to remain prominent. Such areas will not degreen with ethylene but will darken unless fruit are held in very high (ca. 95% RH) humidity after harvest prior to washing and waxing (68). Use of ethephon to enhance rind color break caused an increase in susceptibility to oleocellosis in Washington Navel oranges (71). It was suggested that an increase in fruit rind hydration brought about by stomatal closure induced increased susceptibility to oleocellosis. Zebra-skin, a physiological disorder of tangerines, is influenced also by water conditions of the tree. Losses can be disastrous if fruit are harvested from trees subjected to heavy rains or irrigation following a period of severe drought (72, 73). Susceptible fruit are so fragile that the normal packing processes of washing, drying, and waxing cause the peel to darken over the

segments, particularly in fruit previously degreened with ethylene.

Once fruit are separated from the tree, they are no longer provided with a source of moisture. Loss of water from the fruit after harvest before waxing can occur during delays in handling. This can often lead to a physiological deterioration of the rind near the stem-end called stem-end rind breakdown (68, 74, 75, 76). The disorder is particularly troublesome if fruit are held two or more days at low relative humidities and high rates of air flow between harvesting and waxing. Relative humidities less than 85% during degreening aggravate this disorder (74). The condition is characterized by a collapse of the rind tissue around the button which may extend several centimeters towards the fruit equator. A ring of tissue of approximately 3 mm immediately surrounding the button which is immune to breakdown was observed to lack stomata and to be covered with a heavy deposit of epicuticular wax (77).

Stylar-end breakdown, a physiological disorder of Persian lime, is also enhanced by rough handling at harvest or during packing (78, 79, 80, 81). Rough handling, however, is not the primary cause. Davenport and his co-workers (82, 83, 84, 85) found that rupture of the vesicles in the pulp liberated juice which invaded the rind. The juice passed more easily from the central axis to the rind at the stylar-end than at the stem-end. Fruit turgor associated with thermal expansion of fluid within the juice vesicles increased internal pressure to a magnitude sufficient to rupture some juice vesicles, especially those in larger sized fruit. Stylar-end breakdown in limes can be controlled by maintaining a strict picking schedule so that fruit is harvested before it becomes too large, by controlling post-harvest fruit temperature by hydrocooling in the field and/or assuring that harvested fruit is kept shaded throughout the day, picking fruit with rind oil release pressure of 4.55 kg and greater, or reducing turgor by forced evaporation (84, 85).

Central California Washington Navel oranges are susceptible to a rind staining disorder which is also physiological in nature. The disorder becomes apparent soon after processing and varies from brownish discolorations on the rind to severe cases of surface breakdown. Early in the season well-colored fruit showed more staining than fruit picked green and degreened with ethylene. Fruit harvested late in the season stained more than fruit harvested in early or mid-season. Rough picking practices and prolonged brushing increased rind staining (42, 86).

Mechanical harvesting. Efforts to develop systems to mechanically harvest citrus fruits, particularly oranges, have been in progress for several years. Usually, these systems have been developed to handle fruit destined for processed as opposed to fresh utilization. Some efforts, however, have been made to

utilize mechanically harvested fruit for fresh fruit marketing. Due to the rough handling procedures involved, fruit harvested mechanically are subject to more injuries than are fruit harvested by hand. Increased injuries lead to higher levels of decay (87, 88, 89) and increased moisture loss (90). In addition to injuries, mechanically harvested fruit often have adhering long stems (89, 91), which require removal before fresh utilization. Use of effective fungicides, applied before or immediately after harvest, suppressed decay so that levels were comparable to that in hand-harvested fruit (91, 92).

Fruit harvested mechanically for processed purposes require the use of certain grading techniques at the processing plants (93, 94, 95) to remove culls and trash. Such techniques are generally not needed to such an extent for hand-harvested fruit. Abscission chemicals, which reduce the attachment force of the fruit to the stem, have been studied as an aid to mechanical harvesting (96). The more effective abscission chemicals act by damaging the rind, causing release of ethylene which weakens cells within the abscission layer, resulting in abscission. The technique of loosening citrus fruits with abscission chemicals can influence essential oil composition and degrade the flavor of the orange juice (97, 98). Quantitative analysis of the major volatile compounds of cold-pressed orange oil showed that oils from abscission chemical treated, barely mature, oranges contained less linalool and more α -sinensal, citronellal, dodecanal and valencene than oils from the unsprayed fruit. In oils from mature fruit (99), differences could not be detected and the composition was similar to that of the barely mature chemically treated oranges. It was concluded that abscission chemicals which injure the fruit accelerate maturation (98).

Postharvest techniques and their influence upon quality

Degreening, washing, and grading. The use of ethylene to remove chlorophyll from the rind of citrus fruits in order to expose the orange, orange-red, red, or yellow pigments is practiced extensively. This is particularly true in subtropical citrus regions where fruit maturation usually precedes the low temperatures necessary to remove chlorophyll from the peel. Quality based on eye appeal is enhanced extensively by such a treatment. Understandably but illogically, green color within the rind represents immaturity to consumers and, therefore, they are reluctant to purchase the product. It has been reported that green color is the major reason for low pack-outs in Florida citrus (100). The degreening treatment does not improve eating quality but, on the contrary, it contributes to physiological disorders, senescence, and losses from decay (101). However, because of the consumer's expectations that oranges and mandarins are orange-colored, lemons and grapefruit are

yellow and limes are green, research efforts continue in an effort to improve the color quality characteristic of each cultivar and type.

If performed correctly, degreening does not harm the internal quality of citrus fruit (98, 102). Unfortunately, critical attention to the conditions necessary for proper degreening is not always given. Since degreening never improves internal quality but only the external appearance of the rind, only fruit of proper maturity and internal quality should be degreened. Citrus fruits as they mature on the tree do not produce natural color uniformly, therefore, early in the season, much variability in color exists. In such instances, all fruit, regardless of color, are held in the degreening room until fruit of the greenest color has degreened. Spot picking of fruit with the best color break eliminates the need for excessive degreening time which occurs if fruit of mixed color are harvested. Losses during degreening of tangerines can be reduced by spot picking for color (103). Sorting of fruit on the basis of color after harvest is also another technique available to reduce degreening time (104, 105). Fruit which may regreen on the tree, such as Valencia oranges, are difficult to degreen (101).

There are numerous reviews describing degreening (101, 106, 107, 108, 109, 110, 111, 112, 113). The most common procedure involves continuous metering of ethylene into a room exposing fruit to a concentration of 1 to 20 μl ethylene/l of air for 1 to as many as 3 days. Increased concentrations of ethylene will not shorten the degreening time. Humidities are maintained at levels from about 85 to 95% and temperatures range from 21.1 to 30°C, depending upon geographical areas (114, 115, 116). One exception to this procedure is practiced in Japan where fruit are exposed to a high initial concentration of 1000 μl ethylene/l of air for 15 hours and then removed to ambient conditions (117, 118). Degreening continues for the following 2 or 3 days depending on cultivar. Variations from these procedures during degreening which cause delays in removal of chlorophyll or excessive moisture loss can reduce fruit quality. The most rapid rate of removal of chlorophyll occurs at the higher temperatures, but with relatively little synthesis of carotenoids. A different degreening procedure has been adopted recently in Israel (106, 119). Intermittent exposure of fruit to ethylene and heat in 12 hour cycles at low rates of ventilation caused decreased respiration rates, less peel injury and rot and maintained the fruit at a higher relative humidity. Under these conditions of low ventilation, carbon dioxide concentrations reached levels of 2.5 to 5.0%, which did not interfere with chlorophyll removal as long as oxygen levels were adequate (120). Oxygen concentrations below 10% caused a reduction in the degreening rate (121). Other workers have reported that inadequate ventilation rates allowing an excess of 1% carbon dioxide accumulation will interfere with the degreening process (108, 109).

Quality of lemons reportedly was improved by degreening which increased the juice quantity (122). Ethylene degreening prior to cold storage reduced chilling injury of green-colored grapefruit. However, ethylene tended to increase chilling injury of fully colored yellow grapefruit (123).

Exposure of citrus fruits to ethylene has been shown to increase the incidence of stem-end rot caused primarily by *D. natalensis* (113, 124, 125, 126, 127). Higher than recommended ethylene concentrations (75, 106, 116, 128, 129) and temperature (116) and an increase in degreening duration (116, 125) caused significant increases in stem-end rot. Anthracnose, caused by *C. gloeosporioides*, is a serious disease of specialty citrus hybrids such as Robinson, Lee, Nova, and Page (130, 131), when fruit are exposed to ethylene. Incidence was related directly to length of degreening (130), ethylene concentration (132, 133), and fruit color (133, 134). The occurrence of mold caused by *Penicillium digitatum* Sacc. is significantly reduced by degreening under Florida conditions (135) where the temperature is held at 30°C. Under these conditions, growth of the organism is significantly reduced and lignification of the injured epicarp at the high temperatures and relative humidities is encouraged (136). Once lignification is initiated, entry of the fungus is impeded.

Normally, fruit are not washed before degreening because washing retards the loss of chlorophyll (108). Jahn (137) reported that washing had little effect on chlorophyll loss during degreening but carotenoid synthesis was significantly reduced by washing before degreening. Carotenoid synthesis was reduced by less brushing than was required to clean the fruit, but "over brushing" had little further effect. Washing before degreening interfered with color development in orange and grapefruit more so than lemons and mandarins (138).

Several benefits from washing before degreening would be gained if it were not for the degreening problem. Processes which follow washing, such as grading or color sorting, would be more effective if done before degreening (137). Washing before degreening has been shown to contribute substantially to decay control (131, 138) by removing propagules of *D. natalensis* and *C. gloeosporioides* from the fruit surfaces and, thereby, preventing infection during or following degreening (131, 132).

Damage to citrus peel due to the action of ethylene has been reported, particularly on mandarin types (109, 117), and certain disorders such as oleocellosis (106) and rind staining (42) have been increased. A burn of Temples during degreening (125) has been shown to be suppressed by a sufficiently high relative humidity (75). Under extremely high ethylene concentrations (200 ul/l) and exposure for long periods of time (4 weeks), stickiness of the rind of Marsh grapefruit has been reported (139). Synthesis of natural wax after harvest is stimulated by ethylene (140).

Improved color of degreened fruit was obtained when it was discovered that ethylene in the form of the gas or ethephon stimulated carotenoid synthesis (141, 142, 143, 144) but at temperatures well below 30°C. This high temperature caused rapid chlorophyll removal but did not stimulate continued carotenoid synthesis and actually inhibited accumulation of B-citraurin (142, 143). Cryptoxanthin, B-citraurin, and to a lesser extent, violaxanthin, accumulated in the epicarp at temperatures of 15-25°C. The yellow pigments, trans- and cis-violaxanthin increased less than orange or reddish-orange pigments. Levels of ethylene required for optimum carotenoid synthesis decreased below 10 $\mu\text{l/l}$ of air as temperatures were lowered from 30°C to 15°C at 5°C increments (143). Ethylene caused some increase in respiration rate, abscission of the calyx, and an effect on aroma of the fruit, but no effect on percentage soluble solids, acid, or color of the juice (143). Stimulation of carotenoid synthesis could also be achieved with postharvest application of ethephon (142, 144). To achieve the improved fruit color resulting from carotenoid synthesis, fruit had to be held longer than the 1 to 3 days normally needed to remove chlorophyll at higher temperatures (142, 143, 144). Unfortunately, wastage due to decay can be excessive during periods of 8-10 days or more required for color development under commercial conditions.

In an effort to reduce degreening time with ethylene or to replace its use, postharvest applications of ethephon either alone or in combination with ethylene have been evaluated (14, 145, 146, 147, 148, 149, 150). In certain instances, postharvest applications of ethephon to green-colored fruit to achieve degreening during transit would be desirable. Results with this procedure may be inadequate since waxing in some studies and with certain cultivars has reduced the degreening action of ethephon (146, 149, 151). Ethephon has had no appreciable effect on changes in percentage juice, citric acid, total soluble solids, vitamin C or pH (152). A concentration of 5000 μl ethephon/l caused rind injuries to lemons and delayed degreening of Clementine mandarins and Shamouti oranges (145). In general, postharvest applications of ethephon have not proved more effective or practical under commercial conditions than the standard ethylene degreening practice.

To remove the green color from lemons, the standard Florida practice is to hold unwashed fruit (washing retards color development) at 15.6°C and high relative humidity until the chlorophyll is removed and the yellow color predominates (126). This procedure requires 2 to 3 weeks during which time juice yield and citric acid increase (153). In an effort to speed up the process, ethephon or standard degreening practices have been used but these usually enhance decay, particularly stem-end rot (126, 148, 150, 153). Degreening was achieved with standard degreening practices if mature fruit were treated with an effec-

tive fungicide before exposure to ethylene to control decay (154, 155).

Chemicals, particularly 2-(4-chlorophenylthio)-triethylamine (CPTA) and some related compounds have been applied postharvest to citrus fruit to enhance carotenoid synthesis (22, 26, 151, 152, 156, 157, 158, 159). The material CPTA, which has been evaluated most extensively, principally stimulates the red lycopen carotenoid which is not a normal pigment for citrus. Therefore, its practical use appears limited (157). The compound 2',4'-dichloro-1-cyanoethanesulphonanilide applied pre- or postharvest stimulates rapid and uniform removal of chlorophyll (26, 160) if fruit are subjected to light after treatment. In darkness, it does not serve as a degreening agent, but it does interfere with ethylene degreening. Since its mode of activity appears somewhat different to that of ethylene, it is speculated that a degreening system might be developed without the detrimental senescence action associated with ethylene (26).

One of the initial processes in the handling of citrus fruit on the packing line is to remove fruit unsuitable for fresh consumption diverting it to processing. Generally, those fruit removed because of size, color, or blemishes have little influence on the quality of processed products. However, it has been suggested that early season small sized fruit of less maturity or late season oversized fruit of poor quality could affect the processed product (161).

Decay control techniques. Fungicides are commonly applied to citrus fruits to protect them from decay caused by numerous fungi. Unlike many fruits, healthy portions of decaying citrus fruits are normally not salvaged, and any decay of a fruit means that the entire fruit is discarded. Postharvest fungicides are utilized to improve keeping quality. These chemicals appear to have little influence on internal quality or nutrition. At least if they do, the effect is so subtle that research within this area has not been justified. Several extensive reviews on the subject of postharvest fungicides have been published (162, 163, 164, 165), as have specific fungicide recommendations to control citrus decay (166, 167, 168).

Application techniques can influence the efficacy of postharvest fungicides and, thus, alter the keeping quality of treated fruit. Application of sodium ortho-phenylphenate (SOPP) in a foam washer with an exposure time of only 15 to 20 sec is not as an effective method as a soak or drench treatment requiring 2-4 min (167). However, if proper pH control is not maintained, fruit may be burned with the soak or drench treatment (169, 170, 171). Applications of SOPP in wax were less phytotoxic (172). Within the last 14 years, development of the benzimidazoles, thiabendazole [2-(4'-thiazoly) benzimidazole] and benomyl [methyl 1-(butylcarbamoyl)-2-benzimidazole-carbamate], has led to the availability of fungicides with high

levels of activity against the major decay pathogens. Literature pertaining to the application of the benzimidazoles and techniques which alter their efficacy has recently been reviewed (173). Application of the benzimidazoles by suspension in water emulsion wax or by dissolving in a hydrocarbon-soluble (solvent) wax has not usually provided as good decay control as when the materials are applied in water. Nonavailability of the fungicide at the infection site because of incorporation within the wax and variable coverage with the wax application, particularly under the calyx for stem-end rot control, have been suggested as reasons for reduced decay control. Less fungicidal activity by benomyl may occur in alkaline water waxes where the material degrades to methyl 2-benzimidazole carbamate (MBC) and 1,2,3,4-tetrahydro-3-butyl-2,4-dioxo-5-triazino [a] benzimidazole (STB) (174, 175), which has very weak, if any, fungicidal activity (164). Applications of TBZ, benomyl and MBC in wax for sporulation control of *P. digitatum* to reduce soilage (surface contamination of healthy fruit by mold spores) have shown that benomyl is the most effective of the three materials (167, 175, 176). Benomyl penetrated the peel more efficiently than MBC, forming a fungistatic barrier in the epicarp that prevented eruption of the hyphae of *P. digitatum* through the epidermis (175). Preharvest applications of benomyl to Valencia oranges for the control of post-harvest decay significantly reduced aging (shriveling of the rind at the stem-end) (52).

Vapor-phase fumigants are used for citrus decay control, diphenyl being the most effective and commonly used material. The undesirable odor imparted to treated fruit dissipates within a few days after removal of the fruit from the diphenyl atmosphere.

Control of stem-end rots caused by *A. citri*, *D. natalensis*, and *P. citri* with postharvest applications of the growth regulators 2,4-D and, occasionally, 2,4,5-T is attributed to preservation of the green button and prevention of abscission (106, 177, 178, 179, 180, 181, 182). Freshness and retardation of color changes associated with senescence are benefits of the treatment (183, 184). Gibberellic acid applied after harvest was also reported to reduce decay of lemons (183), but its primary effect is to retain chlorophyll (183, 185). Total soluble solids, acidity and ascorbic acid were not significantly influenced by 2,4-D (184) or GA (183, 184).

Treatments of citrus fruits to control decay fungi which leave no chemical residues would be desirable. Eradication of incipient infections of decay fungi with heat treatments and gamma radiation have been tried. Immersion of citrus fruits in water at 48°C for 2-4 min to control species of *Phytophthora* has been shown to physiologically weaken lemons, in some instances, and increase their susceptibility to other pathological diseases during storage (186). Decay caused by *P. digitatum*, *P. citri*, and *D. natalensis* was reduced by treating oranges at

53°C for 5 min (187, 188), but physiological breakdown of the rind was increased. Cold turgid lemons required slight dehydration before immersion to reduce oleocellosis (189). Treatment at elevated temperatures to enhance decay control with SOPP increased the incidence of oleocellosis and darkened injuries of the rind (169). Use of gamma rays to destroy pathogens in established lesions within the fruit has generally been unsuccessful. Dosages required to eradicate or retard certain pathogens, particularly the stem-end rot fungi *Diplodia* and *Alternaria*, generally caused adverse effects to the fruit. These were manifested as damage to the rind (190, 191, 192), off-flavors (191, 193, 194) and loss in internal quality and weight (191, 195). Respiration rates (191, 195) and ethylene evolution (196) are increased by gamma radiation treatments. There is a marked loss in ascorbic acid in lemons treated with 200 Krad or above (195), but not in Washington Navel oranges treated with 200 Krad or less (191). A thorough review of the effect of gamma radiation on citrus fruits is available in the literature (194).

Waxing. So-called wax coatings are normally applied to citrus fruits to replace natural wax removed in washing, to impart gloss to increase consumer appeal, and to reduce shrinkage from loss of moisture. Both natural waxes and synthetic resins are used and are applied as aqueous emulsions or (for the resins) as solutions in an organic solvent. Some additional effects from wax applications have been observed. Wax coatings lowered internal oxygen and raised carbon dioxide levels (197, 198, 199, 200, 201) and thicker than normal applications may cause ethanol accumulation (197, 202, 203) and off-flavors. A thickness of coating which reduced the weight loss to 50% of that of untreated fruit produced internal anaerobic conditions and off-flavors (197, 204). Ethanol build-up in juice and off-flavors occurred after multiple coatings of water wax or solvent-type wax; less ethanol accumulation and no off-flavors were noted in polyethylene-coated fruits (203). Reductions in chilling injury of grapefruit (205, 206), pitting of Valencia oranges (207), black spot (*Guignardia citricarpa* Kiely) of Valencia oranges (208), decay of lemons (183), and an increase in chilling injury of limes (209) has been obtained with wax applications. Waxing with aqueous emulsion waxes has been shown to retard degreening (210).

Fumigation. Many countries importing citrus fruits require that the fruit be treated to eradicate eggs, larvae, and pupae of fruit flies so as to prevent their introduction into uninfested areas. Treatment for the eradication of at least 5 species of 3 genera of fruit flies has been reported. Ethylene dibromide (EDB) and occasionally methyl bromide, generally applied as fumigants, have been utilized most extensively for this purpose. If careful handling and proper treatment are not

followed, rind of citrus fruits can be discolored or pitted by the fumigation treatment (211, 212, 213, 214, 215, 216, 217, 218). Fumigation damage resulting in rind injury may be associated with prior precooling before treatment (217) or with inadequate ventilation after fumigation (212, 214, 217, 219). Refrigeration should be delayed for at least 24 hours after treatment (217). Injury to Marsh grapefruit and Shamouti and Valencia oranges was due to the persistence of residues of the fumigant in the fruit peel (212). The desorption rate during aeration increased with temperature. Incidence of peel injury was highest in fruit stored at low temperature or wrapped in polyethylene bags, probably due to prolonged action of the EDB residues on the peel. Storage of fumigated fruit in an atmosphere containing an increased concentration of carbon dioxide delayed the appearance of damage. Fumigation injury was enhanced by the presence of diphenyl impregnated in pads (217). Ethylene degreened grapefruit were slightly more sensitive to EDB fumigation than non-degreened fruit (220). Respiration of lemon and orange fruits was stimulated by EDB exposure and was proportional to the dosage level (221). Treated fruits also evolved more ethylene, particularly those exhibiting injury (211). The effect of chamber load on EDB absorption and rind injury showed that reducing the loading rate from 30 to 15% increased EDB absorption considerably and often caused development of severe rind injury (215). Increasing loading rates from 45 to 60% had little effect on absorption of EDB by the fruit. Nutritional value of lemons, as determined by percentage juice and soluble solids, citric acid, pH, and ascorbic acid, was not affected by fumigation (221). The application of TBZ to citrus fruit in the wax coating either before or after fumigation markedly reduced the incidence of EDB peel injury (222). Applications of TBZ in a water suspension or in a water suspension followed by the wax coating which did not contain the fungicide did not effectively reduce the incidence of the injury. EDB reportedly was stable and nonreactive with the constituents of the tissues of Valencia and navel oranges, and lemons (219).

Handling Practices. Methods used in the packinghouse to prepare fruit for the fresh market may also influence the shelf-life and internal quality. Processing Dancy tangerines on the packinghouse line curtailed the subsequent life of the fruit, particularly when degreened (103). Rind breakdown in oranges was shown to increase with processing (223). Washing, color-adding, and waxing treatments had little effect upon the total soluble solids, total acids, or vitamin C content of the juice. Taste tests, however, indicated that flavor was never improved, and in some lots receiving the full packinghouse treatment, taste was unmistakably impaired (223). Dropping of Florida grapefruit during harvesting (64) or of Satsuma mandarins during the packing process (224) was shown to decrease firmness during

storage. The respiration rate of citrus fruit is increased by rough handling (81, 201, 224, 225), and handling also hastened yellowing of Persian limes (81). In the case of dropped Satsuma mandarins held in storage (224), the ratio of dehydroascorbic acid to total ascorbic acid in the pulp increased in proportion to the number of times the fruit were dropped. Most types of mechanical injury to mandarins were produced upon dropping impact, compression, and abrasion (226). Two major factors controlling breakdown of juice sacs were dropping height and compression (227).

Several postharvest treatments to citrus fruits have been tested in an effort to improve the quality of the extracted juice. Bruemmer and Roe subjected citrus fruits to anaerobic conditions for periods of 20 to 32 hours at 32.2 to 43°C (228, 229). This treatment reduced the titratable acidity and increased the Brix-acid ratio by about 10%. The decrease in acidity was accompanied, however, by a 20-fold increase in ethanol (229). Since the soluble solids-acid ratio is a major criterion of citrus juice quality, this procedure, if perfected, could allow earlier harvesting of fruit and a more consistent supply of fruit during the processing season. Bitterness of products from navel oranges, lemons, and grapefruit is related to limonin content. A 3-hour treatment of fruit with 20 ul ethylene/l of air lowered the limonin content, reduced bitterness, and the juice was judged more palatable than juice from untreated fruit (230).

Storage and Quality of Citrus Fruits

Cold Storage. Citrus fruits may be stored to provide a source of fruit at times other than during the normal harvesting season, to hold fruit until a market is available, or while fruit is in transit to a distant market. Unlike deciduous fruits, citrus does not undergo rapid chemical or physical changes after harvest. Citrus fruits are non-climacteric, hence, they do not ripen or improve in quality after harvest. The only exception may be the lemon, which is picked hard and green, and which increases in juice and becomes yellow during normal storage periods. If citrus fruits are stored under proper temperatures, humidities, and atmospheres, and are marketed after a proper duration of storage, the quality of the fruit does not differ greatly from that which existed at harvest. Storage of fruit at lowered temperatures reduces fruit respiration and rate of growth of decay pathogens with a resulting proportionate increase in fruit storage life. Since numerous reviews on the subject of changes in citrus fruits during cold storage have been written (1, 231, 232, 233, 234, 235, 236, 237), discussion of the topic will be limited primarily to some of the more recent studies.

Some of the changes characteristic of maturation, notably

decreasing acidity, continue after storage. The fruit begins to lose weight as soon as it leaves the tree and there is generally a loss of soluble solids and ascorbic acid, on the basis of the original fresh weight of the fruit. If the weight loss is excessive, these constituents may actually increase in concentration (233). However, some quite significant losses in ascorbic acid have been reported, particularly with lemons (238) and tangerines (239).

Quality of Valencia oranges stored for 12 and 18 weeks at 15 and 5°C, respectively, was compared to similar fruit left on the tree (240). Storage fruit was definitely of better quality. Color was better, since regreening which occurred in fruit on the tree did not occur in storage. Granulation and stem-end browning also developed in fruit held on the tree but not in storage fruit. Cool-stored fruit was also higher in Brix, Brix/acid ratio, and ascorbic acid. Loss of flavor in storage after 24 weeks at 15°C was associated with a rapid decline in Brix/acid ratio, ascorbic acid, polyphenols, and flavonoids of the juice. Valencia oranges stored at 3.3°C for 6 months decreased in acidity, increased in total soluble solids, and varied in ascorbic acid content (241). Citric acid was the main acid in juice, malonic and malic acids predominated in the rind of Shamouti oranges stored 3 months at 17°C (242). With aging, malonic, succinic and adipic acids increased in rind and juice, but citric and malic acids decreased in the juice. The authors (242) suggest that malonic acid could serve as an indicator of fruit tissue senescence in storage, because accumulation of the acid within the rind begins at fruit maturity, and increased three-fold during three months of post-harvest storage. It has been recently suggested (243) that ethanol may also be an indicator of fruit conditions since it showed greater changes during storage and subsequent holding than did acetaldehyde, total soluble solids, titratable acid, or pH. Ethanol increased more in grapefruit stored for 3 months at 1.0°C than at 4.5 or 10°C. In oranges, ethanol increased more at 10°C than at 4.5 or 1.0°C. Ethanol content in juice of Hamlin, Pineapple and Valencia oranges and Marsh grapefruit increased with decreasing oxygen (O₂) concentration in CA storage and with wax applications (202). The ethanol content of juice of Marsh grapefruit increased with increasing carbon dioxide (CO₂) concentrations from 10 to 30%, with length of time of exposure to CO₂, and with total time of storage. The acetaldehyde content of the juice, although much lower than the ethanol content, increased in a similar manner (244). Under these various storage conditions, the ethanol content was apparently insufficient to affect flavor significantly. The condition of fruit during cold storage (245) was estimated by continuously monitoring the CO₂ produced hourly/kg of fruit and calculating the relative increase in the respiratory intensity. Increases in respiration were due to infected fruit plus the effect of ethylene emanation on metabolism of healthy fruit.

Chilling injury. A major problem with cold storage of citrus fruits is that of chilling injury or pitting, which occurs in chill-sensitive fruits such as grapefruit, lemon, and lime at temperatures of 5-10°C. Eaks (42) reported that limes had to be stored at 5°C to retain green color, but chilling injury developed in about 4-5 weeks. Storage at 10°C eliminated the chilling injury problem, but fruit became yellow-colored after 2-3 weeks. Several techniques in handling citrus fruits, principally grapefruit, have been utilized to mitigate the incidence of chilling injury. Exposure of fruit to high relative humidity (95-100%) for several days before storage (123, 246) or at lower moisture levels (curing) for 1 to 2 weeks (247, 248, 249) have reduced incidence of the disorder. Waxing has also been shown to have a favorable effect (250, 251). Intermittent warming during the storage period has also been successful (252, 253). Chilling injury was reduced by removing fruit periodically from storage at 4.4°C to 21.1°C for 8 hours during an 8-week period (253). Treatment with ethylene before cold storage (123, 249) is another technique for control, however, it was more effective on green-colored fruit (123). Chilling injury of limes was markedly reduced by storage in a partial vacuum at 220 mm of Hg (123), and some influence from growth regulators (benzyladenine, GA, 2,4-D) applied pre- or postharvest has been observed (50). Treatments with high levels of CO₂ (40%) for up to seven days at 21.1°C significantly reduced chilling injury (247). Success using CO₂ treatments in additional studies generally at lower CO₂ levels has been obtained (254, 255, 256, 257, 258). Fungicides applied for the control of decay fungi unexpectedly reduced chilling injury (251, 258, 259, 260). Control with TBZ and benomyl persisted during 16 weeks storage of grapefruit at 2, 5, and 8°C (251). TBZ was more effective than benomyl and treatments were most effective when the materials were incorporated into the wax. The effect of TBZ was enhanced by increasing concentration and residues, but the effectiveness of benomyl was not improved with increased concentrations. Reduced chilling injury of grapefruit with the use of benomyl was also observed by McCornack (261), but use of the fungicide diphenyl significantly increased chilling injury. Fortunately, the benzimidazoles, TBZ and benomyl, are used extensively on citrus fruits for decay control and the mitigation of chilling injury from their application provides an additional benefit. Unfortunately, however, the cause of chilling injury has yet to be defined, and none of these handling techniques provide complete control of this disorder.

Pre-cooling. Pre-cooling, a technique to remove field heat from citrus fruits, is advised since refrigeration facilities within most transit containers do not have sufficient capacity to cool fruits rapidly. Fruit is often delivered from the field at relatively high temperatures. Processes within the packinghouse such as degreening, color-adding, and drying contribute further

heat to the fruit. Pre-cooling can be accomplished by air-cooling or hydro-cooling. Forced air at -2°C for 6 to 24 hours prior to ventilated shipment of Shamouti, Valencia and navel oranges and Marsh grapefruit (262) reduced weight loss during simulated shipment and storage. Pre-cooling of fruit to temperatures below 0°C was not advised because of possible effects on quality. Pre-cooling of Florida oranges (263) initially reduced decay levels but then decay levels increased during simulated shelf-life. Water damage from hydro-cooling caused increased decay of tangerines and grapefruit (264) and oranges (265). Decay after hydro-cooled fruit are allowed to come to ambient temperature, as in markets or homes, frequently exceeded that of non-hydro-cooled fruit (266). Peel injury, particularly of Pineapple oranges, was often increased by hydro-cooling.

Controlled Atmosphere Storage. Use of controlled atmosphere (CA), an atmosphere containing more CO_2 and less O_2 than air, to maintain the quality of citrus during storage has generally been no more effective than cold storage in air. Several reviews of the subject published previously cover results of some of the earlier work (1, 207, 236, 237, 267, 268, 269). Various results have been obtained and indicate that factors other than O_2 and CO_2 concentrations, such as variety, maturity and humidity may be involved. Decay is often enhanced with certain CA storage conditions (268, 270, 271, 272), disorders of the rind may be increased (209, 270, 271, 273) and off-flavors may develop (271, 274). However, some improvement in keeping quality with CA storage has been reported. Valencia oranges held in 15% O_2 -0% CO_2 for 12 weeks at 1°C plus 1 week at 21°C had higher flavor ratings than similar fruit held in other controlled atmospheres or in air (267). Addition of either 2.5 or 5% CO_2 controlled aging. No marked differences were found in total soluble solids, total acid, pH, ascorbic acid, or hesperidin between Valencia oranges from CA storage and those from air (207). Less decay developed following storage of Valencia oranges at 15 or 10% O_2 with 0% CO_2 than when stored in air (275). Pitting (chilling injury) of grapefruit was eliminated by CA storage at 10°C but not lower temperatures (267). Controlled atmosphere storage of Satsuma mandarin increased the proportion of marketable fruit, fruit density, and citric acid concentration. Optimum conditions were 1% CO_2 with 6-9% O_2 (276). Storage of navel oranges at 15°C at high O_2 levels (40-80%) improved the orange color of the endocarp and of extracted juice (277).

Some of the most optimistic results with CA storage have been reported from work with lemons. Biale (278) indicated that lemons may benefit from CA storage. Storage life was prolonged markedly by atmosphere of 5 and 10% O_2 . Respiration rates were reduced, color changes were retarded, and decay, caused primarily by *A. citri*, was reduced. An atmosphere of 6% O_2 without CO_2 was the best treatment for lemons at 15.6°C (272, 279). Levels of 5 to

8% O₂ reduced decay to less than that in air storage (279). The importance of removing ethylene, which may accumulate in storage facilities, to enhance keeping quality was emphasized by some recent studies (280). Valencia oranges were stored at 10°C in 5% CO₂ and 3% O₂ without and with an ethylene absorbant Purafil (porous alumina beads impregnated with potassium permanganate). By keeping ethylene concentrations to less than 0.8 ul/l of air, stem-end decay caused by a species of *Fusarium*, green mold, loss of orange flavor, and development of off-flavors were minimized. Other volatiles present with and without the ethylene absorber apparently had no effect on quality. Ethylene, produced in copious quantities by rots, enhances respiration and senescence of healthy fruit within the storage facility (281). The importance of ethylene removal has been illustrated also in recent storage studies of lemons in Australia (282, 283, 284, 285). Lemons remained in good condition for up to 6 months under an atmosphere of 6-10% O₂ and essentially no CO₂ at 10°C with continuous removal of the ethylene (285). The lower ethylene concentrations significantly reduced the development of green mold (284). Oxygen concentration in the atmosphere was also shown to regulate the effect of ethylene on the rate of green color loss from lemon rind. An atmosphere of 11% O₂ and 6 ul/l of accumulated ethylene caused a more rapid loss of green color than did 5% O₂ and 300 ul ethylene/l of air. Removal of ethylene was useful in maintaining the green color but it was more effective when combined with CA storage. By treating fruit with 2,4-D and GA, both at a concentration of 500 mg/l, optimum results were obtained in an ethylene-free CA. The 2,4-D effectively prevented senescence of the button while the GA retarded the development of the deep yellow color associated with over-maturity.

Attempts to store limes in CA often resulted in scald-like injury to the rind and an increase in the incidence of decay (209, 272). Limes stored in a CA of 5% O₂ with 7% CO₂ maintained acceptable green color, but had low juice content, thick rinds, and a high incidence of decay (286). However, limes were successfully stored when held under low pressure (hypobaric) conditions. Berg and Berg (287) reported limes were still green after 8 weeks at 150 mm Hg and 15°C, whereas, 50% of control fruits turned yellow in 10 days. Spalding (286, 288) observed that Tahiti (Persian) limes retained green color, juice content, and flavor acceptable for marketing and had a low incidence of decay during storage at a low atmospheric pressure of 170 mm Hg for up to 6 weeks at 10°C or 15.6°C and a relative humidity of 98-100%. Check fruits turned yellow within 3 weeks at normal atmospheric pressure. Low pressure storage did not prevent chilling injury of fruit stored at 2.2°C. At 21.1°C, fruit stored at 170 mm Hg remained green and suitable for marketing after 3-4 weeks if treated with TBZ or benomyl for decay control. Removal of ethylene at low pressure was suggested to be involved in retardation of ripening (287), and appears to be critical to

the success of storage of both lemons and limes. Low pressures less than 50 mm Hg were required to retard growth of common postharvest decay fungi (289). Action was fungistatic rather than fungicidal.

Fruit Quality as Influenced by Packaging and Transportation

Packaging. Techniques used in packaging and transporting citrus fruits influence fruit quality. The package and/or container must protect the fruit from mechanical damage and provide a favorable microclimate for the fruit if quality is to be maintained from harvest to consumption. Various reviews on the subject of packaging and transporting citrus have been published previously (250, 268, 290, 291, 292, 293, 294). Therefore, only the major aspects as well as recent developments not previously reviewed will be discussed here.

Relative humidity is critically important to the preservation of quality, both in its influence upon the fruit and upon decay pathogens (295). Humidity within the package or container can vary extensively. Microclimate changes occur due to transpiration, respiration, and to changes in ambient conditions (291, 296, 297). Interactions between fruit and air temperatures when fruit are transferred into and out of refrigerated conditions can have drastic effects on humidity (292). High humidity and poor ventilation in polyethylene bags or shrink-film wrapped paper trays have led to increased amounts of decay (290, 291, 298, 299, 300). Therefore, perforations within the film are required to reduce humidity and CO₂ levels (301). On the other hand, fruit packed in mesh bags can lose moisture excessively and become flaccid and shriveled (300). Decay of only one fruit within a package makes it unacceptable to the consumer, and volatiles from decaying fruit within a polyethylene film bag may produce off-flavors in the juice of sound fruits within the same bag (302).

Films possessing different permeability to O₂ and CO₂ have been used to develop atmospheres and/or humidities which improve quality. Chilling-injury was reduced in grapefruit by packaging fruit under various types of films (206, 303, 304). Some recent success in improving keeping quality of citrus fruits has been obtained by wrapping individual fruit within film of high density polyethylene. Ben-Yehoshua and co-workers (305, 306) markedly delayed deterioration of individually wrapped Shamouti and Valencia oranges, Marsh grapefruit, and Eureka lemons. Peel shrinkage, softening, deformation, and loss of flavor was delayed. Sealed fruit maintained the fresh appearance more than twice as long as conventionally handled fruit. Weight loss was reduced about five-fold. Respiratory activity and ethylene production was also reduced in wrapped and sealed fruit. In a separate study (307), grapefruit wrapped in polyethylene bags lost less weight, maintained optimum color and gloss, and developed less stem-end rot than unwrapped fruit.

Transportation. Deformation of citrus fruit, due to inadequate protection by the container, detracts greatly from external appearance. Over-filling of cartons, so that weight of stacked cartons is supported by the fruit rather than the container, contributes significantly to deformation (294). Problems with malformation occur more frequently in situations where fruit are transported over long distances, such as during export. Hale (308) reported that 33 to 60% of the Florida grapefruit shipped to Japan arrived seriously deformed. Deformed grapefruit were more prevalent in the bottom layer of the carton regardless of the type of carton used. Usually, the larger the fruit, the more seriously the fruit were deformed. By packing fruit in experimental (309) or international standard shipping cartons (310), which were somewhat deeper than the standard 4/5 bushel carton, deformity was reduced significantly since more of the weight was supported by the carton. Condition and appearance of large-sized grapefruit were improved by packing fruit in special tray-pack containers (311). Serious deformation of tray-packed fruit was reduced to an average of 2.7%, compared to 27.9% for fruit shipped in the standard export box.

During handling and shipping grapefruit from South Africa to Europe, percentage juice and rind thickness decreased (34). The percentage of total soluble solids increased and the acid content remained undamaged.

Conclusions

Quality of citrus fruits is altered quite significantly by numerous techniques applied before and after harvest. Since appearance of the fruit is so vital to consumer acceptability, considerable efforts towards improving and preserving the quality of the rind can be expected in future studies. Damage resulting from unnecessary rough handling during harvesting and processing predisposes fruit to increased decay caused by several wound pathogens. Even though effective fungicides such as the benzimidazoles have been developed, these materials can not be expected to cure the damage caused by improper handling. Rather, proper fungicide applications combined with good handling and sanitary techniques will insure citrus fruits of excellent keeping qualities. Problems with fungal resistance and lack of control of minor decays are sufficient to warrant further investigations with postharvest chemicals for decay control.

Cold storage still appears to be the most effective method for preserving quality in citrus fruits except for lemons and limes. Recent studies with lemons have shown beneficial effects of CA storage where ethylene is effectively removed. Similar results have been obtained with limes stored under low pressure (hypobaric) storage.

Recent results in improving keeping quality of citrus fruit by individually wrapping them in high density polyethylene await additional confirmation and application to commercial practices.

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Citrus Juice Processing as Related to Quality and Nutrition

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There is much that can be said in favor of the consumption of fresh fruits and vegetables in the daily diet. In much of the world, citrus is consumed primarily as the fresh fruit, but in the United States processed products are consumed as the major source of citrus in the diet. The main staple of processed citrus juices in the U.S. is frozen concentrated orange juice (FCOJ).

Were it not for the processing of citrus fruits, this rich source of nutritious food, in the forms of juices and drinks, would be available to us for only limited periods of time throughout the course of any year. Processing techniques practiced today in the citrus industry ensure the availability of a continuous supply of citrus juices and their allied products to people in all regions of the United States and, indeed, in many parts of the world.

Our increased knowledge of nutrients in the food supply and how they are affected by processing has led to an increased awareness on the part of processors about the nutritional aspects and qualities of their products, and for a greater desire to improve processing techniques so that the consumer can derive maximum benefits from those nutrients. There has been an increased recognition in the food industry that we have some responsibility for the nutritional quality of our food supply. This awareness of responsibility has led to increased safeguards in processing so that not only the nutritional quality, but also the flavor acceptability is better retained in the processing of natural foods.

The increased awareness on the part of consumers about nutrition has led to an increased demand for citrus juices and products, a demand that is greater today than it has ever been. This has led to a tremendous growth within the citrus industry, and developing nations of the world that have climates suitable for the production of citrus fruits have benefitted tremendously from this consumer demand. Brazil is a prime example. The growth of the citrus industry in Brazil has been a great economic factor in

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the welfare of its people. Brazil today is a major factor in the worldwide supply of citrus products and is second only to the U.S. in the production of citrus. This is evidenced in Table I, which presents data on citrus fruit production by specified countries (1). The overall growth of the citrus industry is, of course, fostered by improved economic conditions in many countries and evolving technologies that permit the production, storage, and shipment of citrus products over long distances. Refrigeration, new distribution methods, and new packaging techniques represent developments without which many people in the world could not enjoy the full flavor and nutrition of citrus products.

The unique and distinctive flavors of the citrus fruits and the general acceptability of these flavors by peoples throughout the world have also been factors contributing to the growth of the citrus industry. Orange flavor is probably the most widely recognized and accepted flavor in the food and beverage industry worldwide. Because of its distinctive flavor and aroma it is used to flavor many foods and beverages and to aromatize many household products.

Grapefruit is less popular than the orange but perhaps more popular than the lemon relative to consumption of the juice. Popularity of grapefruit juice is increasing in many parts of the world, particularly in the United States and in Japan. How much its chimerical image as a dietetic food has contributed to this increasing popularity is not known, but it undoubtedly has been a factor. Chilled and bottled grapefruit juices are growing in popularity whereas the usage of frozen concentrated grapefruit juice continues to grow, but at a slightly slower rate.

Lemon juice has many uses in the food industry that other juices do not have because of its uniquely different composition in relation to other juices, except perhaps lime. Large quantities of lemon juice are used to enhance food flavors and to develop and balance the flavors of many food items, seafood being an outstanding example. Possibly only sugar and salt are used more extensively in the development and enhancement of food flavors. Lemon juice has also gained in popularity because of technological advances that now permit the manufacture of concentrated juices and the production of a frozen concentrate for lemonade.

The flavor of lemon, contributed by the peel oil, is probably second only to orange flavor in overall popularity. The growth in market for the powdered soft drink mixes and the fruit drink mixes, particularly for lemon-flavored products, has increased the demand for lemon oil. Added to this is the increasing demand for lemon oils for use in the carbonated and noncarbonated soft drinks that are increasing in popularity worldwide.

TABLE I

Citrus Fruit: Production, by Selected Countries,
of Principal Types, Crop Years 1976-77 through 1978-79 (1)

Country	Crop Years		
	1976-77	1977-78	1978-79 <u>2/</u>
1,000 Metric Tons <u>1/</u>			
<u>Oranges and Tangerines</u>			
United States	10,144	9,256	8,725
Brazil	6,087	8,205	7,256
Japan	3,575	4,119	3,663
Spain	2,466	2,514	2,473
Italy	2,258	1,942	1,699
Morocco	784	1,055	992
Israel	968	949	975
Argentina	990	925	926
Mexico	1,283	750	783
Egypt	840	747	780
Turkey	671	735	780
Greece	533	455	629
South Africa	463	591	583
Australia	384	393	395
Cyprus	100	109	113
Chile	45	47	48
TOTAL	31,591	32,792	30,820
<u>Lemons</u>			
United States	896	900	758
Italy	792	800	600
Brazil	371	363	367
Argentina	320	280	267
Turkey	278	280	250
Spain	220	313	239
Greece	190	194	175
TOTAL	3,067	3,130	2,656

1/ One metric ton is equivalent to 2204.6 pounds.

2/ Preliminary.

The Florida Crop and Livestock Reporting Service

TABLE II

U.S. Production of Oranges
Years 1973-78 (1)

Crop Year	Utilization of Production 1000 Metric Tons			% Tons Processed
	<u>Fresh</u>	<u>Processed</u>	<u>Total</u>	
1973-74	1613	6900	8514	81.0
1974-75	1951	7339	9290	79.0
1975-76	1803	7717	9519	81.1
1976-77	1680	7886	9567	82.4
1977-78	1598	7059	8657	81.5
1978-79*	<u>1451</u>	<u>6854</u>	<u>8306</u>	<u>82.5</u>
AVERAGE	1683	7292	8976	81.2

*Estimate

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1 Processing

Technological developments in high vacuum evaporation techniques have been responsible for the rapid growth of the domestic citrus industry. These techniques were developed and refined, for the most part, during World War II and they made possible the manufacture and production of many perishable foods and medicines. Most notable for the domestic citrus industry was the development of frozen concentrated citrus juices which was made possible by the development of these high vacuum evaporators

Frozen concentrated orange juice began to capture a real segment of the citrus market in 1948, and since then, its presence has been a dominant contributing factor to the increasing per capita consumption of citrus juices worldwide.

Processed orange products accounted for the usage of about 81% of the domestic orange crop between the years 1973 and 1978, as can be seen in Table II. Frozen concentrated orange juice in that period was by far the major product of the U.S. citrus industry, which is concentrated in 4 states; Florida, California, Texas, and Arizona, with Florida being the dominant factor in the industry.

About 94% of the Florida orange crop went into the production of orange juice products during the 6-year period, 1973-1978, and frozen concentrated orange juice accounted for approximately 81% of that usage. About half of the orange crop of Texas and about 40% of the Arizona crop were utilized in processed products, but only about one-third of the California crop was so utilized. The major portion of the latter crop went to the fresh fruit market. These data are summarized in Table III.

TABLE III

U.S. Production of Oranges by Region
6-Year Average 1973-1978 (1)

<u>1000 Metric Tons</u>			
<u>State</u>	<u>Processed</u>	<u>Total Production</u>	<u>% Processed</u>
Florida	6614	7069	93.6
California	500	1550	32.3
Texas	129	235	54.9
Arizona	49	122	40.2
TOTAL	7292	8976	81.2

The Florida Crop and Livestock Reporting Service

Similar trends to those noted above exist in the domestic

TABLE IV

U.S. Production of Grapefruit
Years 1973-1978 (1)

Crop Year	Utilization of Production 1000 Metric Tons			% Tons Processed
	<u>Fresh</u>	<u>Processed</u>	<u>Total</u>	
1973-74	1023	1416	2439	58.1
1974-75	1038	1231	2270	54.2
1975-76	1193	1390	2583	53.8
1976-77	1034	1716	2750	62.4
1977-78	1101	1646	2747	59.9
1978-79*	<u>1038</u>	<u>1452</u>	<u>2490</u>	<u>58.3</u>
AVERAGE	1071	1475	2547	57.9

*Estimate

The Florida Crop and Livestock Reporting Service

grapefruit market, but that total market is only about 30% as large as that for oranges as seen in Table IV. Almost two-thirds of the Florida grapefruit crop goes to the production of processed products with frozen concentrated grapefruit juice accounting for about 35% of the processed juice. Chilled grapefruit juice accounts for about 12% of the processed juice, and that market segment is growing along with the bottled grapefruit juice market. Of the grapefruit produced in the other U.S. growing regions, California, Texas, and Arizona, more than half go to the fresh fruit markets. About 46% is processed. These data are shown in Table V.

TABLE V

U.S. Production of Grapefruit by Region
6-Year Average 1973-1978 (1)

<u>State</u>	<u>1000 Metric Tons</u>		
	<u>Processed</u>	<u>Total</u>	<u>% Processed</u>
Florida	1173	1894	61.9
California	93	200	46.5
Texas	167	375	44.5
Arizona	<u>42</u>	<u>78</u>	<u>53.8</u>
TOTAL	1475	2547	57.9

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Table VI shows the average amounts of the Florida orange and grapefruit crops that went into processed products during the five-year period from 1973-1977. Frozen concentrates accounted for the major portion of the processed orange juice and about one-third of the processed grapefruit juice. Chilled orange juice in bottles and in dairy cartons accounted for a significant portion of the processed Florida orange crop and this is presently the fastest growing segment of the market. Chilled grapefruit juice is a growing market, but bottled, shelf-stable grapefruit juice is also experiencing major growth at the present time. Chilled grapefruit juice accounted for about one-eighth of the processed juice; but, canned and bottled grapefruit juices accounted for a major portion of the processed grapefruit juice as can be seen in Table VI.

TABLE VI

Processed Florida Citrus Products
6-Year Average 1973-1978 (1)

Product Category	Gallons Produced (000's)	Fruit Utilization (1000 Metric Tons)	% Of Processed Oranges	% Of Processed Grapefruit
Frozen Concentrated Juice (Reconst. Basis)				
Orange	684,419	5644	81.0	
Grapefruit	40,912	405		34.5
Tangerine	4,700			
Blended	28			
Chilled Juice				
Orange*	125,517	978	14.0	
Grapefruit	16,244 (est.)	144		12.3
Canned Juice				
Orange*	40,393	278	4.0	
Grapefruit	66,328	588		50.0
Tangerine	68			
Blended	5,861			
Fruit Sections				
Orange	64	21	0.3	
Grapefruit	7,639	38		3.2
Blended	465			

*Includes Temples, Tangelos, and Honey Tangerines

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1.1 Juice Extraction

Citrus fruit is delivered to a processing plant in truckload quantities of 20,410 to 21,430kg or 20.4 to 21.4 metric tons. The fruit is unloaded, inspected for maturity and graded to remove unwholesome and damaged fruit, after which it is conveyed to fruit bins for storage. Fruit from the bins is washed with a detergent in a rotary brush washer, rinsed, then inspected and graded a second time to remove unwholesome fruit.

Juice extractors differ in design, but all are fast, rugged, easy to clean, and adjustable to accommodate fruit of different sizes. Prior to the invention of automatic extractors, the rotary juice press was in common use, and is still used commercially in many parts of the world, principally Italy, Spain, & South America.

The FMC In-Line Extractor is widely used in the domestic industry, most particularly in Florida, because it can effect simultaneous recovery of both juice and oil. A five-headed extractor can process from 325 to 500 fruit/minute. The extractor consists of a bottom cup, into which the fruit is fed, and an upper cup that meshes with the bottom as circular plugs are cut from the top and bottom of the fruit. The fruit in the bottom cup is compressed as the upper cup descends and juice and other fruit components are forced through the bottom plug into a strainer tube. The contents of the strainer tube, rag, seeds, and cell sacs, are squeezed between the top and bottom plugs resulting in almost complete extraction of juice and, in essence, a first-finishing operation since the plug (seeds, pulp, and peel) is separated from the juice. As the fruit is squeezed in the cup, peel oil expressed from the flavedo and small pieces of peel are washed into a conveyer by a water spray that surrounds the extractor cup. The valuable oil is recovered from the oil/water slurry.

Several types of Brown extractors are used in the citrus industry throughout the world. The Model 400 produces a juice that is low in peel oil content and high in juice quality. The fruit is halved and the juice removed by a rotating reamer that exerts pressure to effect extraction.

The Brown Model 700 Extractor operates in a manner similar to the Model 400 and produces juice of the same high quality with low oil content. It expresses the juice from about 700 fruit/min. compared to the 350 fruit/min. that can be processed by the Model 400.

A more sophisticated extractor, the Brown Model 1100, accepts three parallel lines of single-file fruit, and has a processing capacity of almost 11 metric tons of fruit per hour. It produces maximum juice yields. Fruit entering the extractor is halved by a stainless-steel knife and each half passes between a stainless-steel grid and a rotating disc. The juice is expressed in two stages equivalent to a light extraction (low pulp/low oil) and a hard extraction (higher oil/higher pulp), then flows to the bottom of the collector where it can be divided into two fractions.

The stainless-steel grid provides a coarse first-stage finishing operation simultaneously with the extraction. The juice flows through outlets at the bottom of the collectors and is conveyed to finishers.

1.2 Finishing Operations

In the finishing operation, seeds are removed from the juice and the pulp content is lowered. As with extractors, finishers vary in design. The two types most commonly used are the screw-type and the paddle-type. In both designs, separation is accomplished by a cylindrical perforated screen. Juice and a controlled amount of insoluble solids or fine pulp pass through the screen while the remainder of the solids is discharged at the end of the finisher. The size of the screen perforations determines the size of the solid particles that remain with the juice. The juice extractor and finisher are employed in tandem to control the characteristics of the processed juice, but they also affect the juice yield and quality. The juice characteristics controlled by these operations include the pulp content and size, and oil content.

The finished juice is conveyed to blend tanks at which time the acidity and soluble solids level may be determined. If necessary, the juice can be deaerated and deoiled, dependent upon the product to be produced.

1.3 Evaporation

According to Cook (2), the first commercial orange concentrate was produced in Florida in 1938 on a low-temperature (20°-25°C) evaporator with 13 stages that operated under high vacuum.

Most of the evaporators used in the state of Florida prior to 1947 utilized high temperatures and long residence times. These evaporators operated somewhere between 48.9° and 82.2°C (120°-180°F). Fruit solids remained in these evaporators for a minimum of 30 minutes; hence, the products produced on such evaporators were of poor quality and exhibited a strong heat processed flavor.

In 1946, the first commercial frozen concentrated orange juice was produced in a falling-film type evaporator operated at low temperature and high vacuum. This evaporator, installed by the Minute Maid Company (now The Coca-Cola Company Foods Division) employed a large steam jet pumping system to remove water vapor at high enough vacuum to maintain an operating temperature of about 18.3°C (65°F). The juice stayed in the evaporator for a long period of time, but the concentrate produced was far superior to that produced in high-temperature evaporators.

Low-temperature evaporators of the falling-film type were heavily utilized in the citrus industry through the 1950's and

and early 60's. The first high-temperature short-time evaporator was installed in Florida in 1959. This was a double-effect two-stage unit that employed some recirculation of steam. This research into evaporation principles led to the development of the present units that are used almost exclusively today in the domestic citrus industry. These units are known as TASTE (thermally-accelerated short-time evaporation) evaporators. Most of these units employ a single pass of juice, and they are composed of four to six effects with six or seven stages. The water removal capacity is normally 18,000 to 23,000 liters per hour, but some of the new units being constructed have water removal capacities of nearly 41,000 liters/hour.

Figure 1 illustrates the operating principles of a TASTE-type evaporator (2). Juice passes through the preheaters and is heated to the temperature of stabilization, about 205°-210°F (96.1°-98.9°C), to destroy enzyme activity. After stabilization, the juice passes through the nozzle at the top of the first stage where it flashes to a lower temperature, one that corresponds to the pressure at that point. The resulting mixture of juice and vapor is projected into the tube bundle in the first stage where further evaporation of water occurs as the juice passes down through the tubes.

This is not a falling-film evaporator because the liquid (juice) is mostly suspended in the vapor, and heat transfer is to the turbulent mixture. The exit velocity of the juice from the tubes is on the order of 20 to 100 meters per second (2).

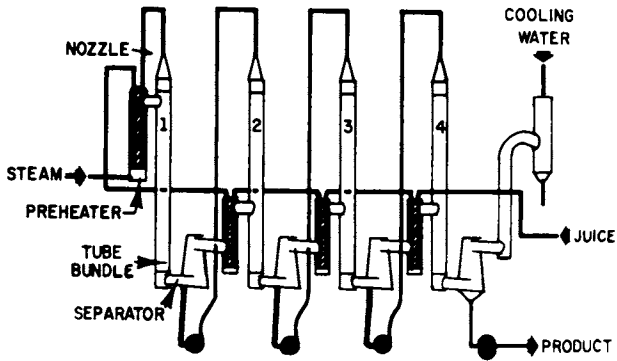
As it exits from the tubes, the liquid and vapor mixture travels into a centrifugal-type vapor separator, and the liquid then flows down the suction line to a heavy-duty pump.

On being pumped to the next stage, the liquid flashes through the nozzle and the mixture travels down the tube bundle as it did in the first stage. Vapor from the first-effect separator provides the heat for evaporation in the second stage.

The juice passes through additional stages, normally about seven in all, and after the last stage, the juice enters a chamber where it is flash cooled to about 10°C (50°F). After flash cooling, the concentrate, which will be at 65°-68°Brix, is pumped into drums or to a holding tank in one of the newly constructed tank farms that are becoming more prevalent in the domestic citrus industry.

In a four-effect evaporator steam is put into the first effect, where an effect relates to vapor flow, and the heat from that steam is used four times before condensation occurs in a barometric condenser. In theory, a four-effect evaporator will remove four liters of water per kilogram of steam usage, but in actual practice that water removal is about 3.4 l per kg of steam. Heat losses and the change in the heat of vaporization with temperature account for the difference.

A typical TASTE evaporator in use in a citrus plant is shown in Figure 2. The Coca-Cola Company Foods Division operates three



Institute of Food Technologists

Figure 1. Flow diagram for TASTE evaporator (2)



Figure 2. TASTE evaporator (courtesy of George Craddock)

citrus processing plants in the state of Florida, and in these plants are eight TASTE evaporators dedicated to the production of orange juice concentrate. Additional evaporators of the same type are also used for by-product production, e.g., citrus molasses and washed pulp solids. The eight evaporators used for the production of orange juice concentrate have a total rated capacity for water removal of 170,000 l per hour. The largest of these evaporators is rated at 36,400 l per hour of water removal.

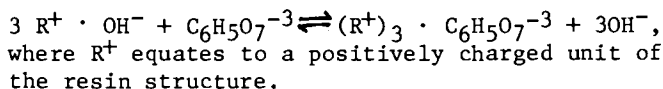
Berry and Veldhuis (3) recently presented a comprehensive treatment of evaporators, and the reader is referred to that article for a more in-depth review.

1.4 Ion-Exchange Processing

Acid removal from citrus juices was first reported by Kilburn and Drager (4) in the early 1960's. They employed electro-dialysis to remove citrate ion from citrus juices. Later, Berry and Wagner (5) used calcium hydroxide for precipitation of citrate in citrus juices.

A more recent process, i.e., acid reduction by anionic ion exchange, was developed at the Research and Development Laboratories of The Coca-Cola Company Foods Division in Plymouth, Florida.

Removal of citrate ion by an anionic ion-exchange process can be accomplished by exchange with hydroxyl ion and the subsequent formation of water, which is a component of juice and which can be removed by evaporation; hence, it should be preferable to a method that relies on the addition of a neutralizing substance to a citrus juice. The ion-exchange process is illustrated in the following equation:



The ion-exchange resin employed in the acid reduction process is weakly basic and is approved for food use as prescribed in the food additive regulation 173.25(a)(14) in Title 21, Code of Federal Regulations (21CFR) (6).

Because the anionic resin is weakly basic, the retention of stronger acids is favored. As a result, when processing orange juice, the retention of citric acid is favored with respect to the weaker organic acids, ascorbic and folic, which are well-recognized nutrients in orange juice. Also, mass action favors the removal of citric acid.

The process permits the treatment of either bulk concentrate or freshly extracted juice. Freshly extracted juice is first stabilized at 175°-180°F (79.4°-82.2°C) then centrifuged in a high speed centrifuge to effect a pulp reduction to 2% to inhibit development of excessive back pressures in the column due to plug-

ging. This pulp can be re-added to the juice stream following acid reduction in the ion-exchange column. Bulk concentrate must first be diluted to about 15°Brix after which pulp reduction is accomplished by centrifugation. Stabilization of the dilute concentrate is not necessary because of its prior stabilization during concentration.

Acid reduction of orange juice is effected by downflow passage through the resin. Juice is passed through the column until the eluate (reduced-acid juice) drops to a pH below 4.6 as monitored by a pH meter. This method assures minimal loss of ascorbic acid.

The column eluate is discharged into an evaporator feed tank where its pH is adjusted to a maximum of 4.6 through the addition of freshly extracted, but untreated, juice or concentrated orange juice. At this time the pulp removed in centrifugation can also be re-added. This adjustment of pH ensures that no growth of pathogenic organisms can occur, and studies by independent laboratories have confirmed this finding.

Following pH adjustment of the acid-reduced juice, it is concentrated to 65°Brix in a TASTE evaporator.

1.5 Pasteurization and Packaging

The purpose of pasteurization, as it is practiced in the domestic industry today, is to destroy spoilage organisms, inactivate enzymes, or both. Heating to temperatures of only 150°F (65.6°C) will destroy most spoilage organisms but some heat resistant molds may require pasteurization temperatures as high as 210°F (98.9°C) for control.

Citrus juices that are pasteurized at the lower temperatures, 65-66°C, can undergo clarification, i.e., a process of separation that results in a lower layer of liquid and sediment and an upper layer of clear liquid. This process is brought about by the natural enzyme, pectinesterase, that occurs in citrus fruits. Studies have shown that processing of the juice at temperatures of 170-210°F (76.7-99°C) for a fraction of a second to 40 seconds will destroy the pectinesterase activity in citrus juices (7-10). The temperature necessary to stabilize the juice is pH dependent. Juices at higher pH require higher temperatures for stabilization. With the new high-temperature short-time techniques and equipment, stabilization can usually be effected in a fraction of a second. Flash pasteurization can be accomplished in either a plate-type or a tube-type heat exchanger.

Chilled juices, both orange and grapefruit, are increasing in popularity and, indeed, this market segment is presently the fastest growing in the industry. These products are pasteurized, cooled, and filled into paper cartons lined with a plastic or aluminum foil laminated with a plastic.

The chilled juice market experienced much of its initial growth through dairy processing and delivery systems, but today

much of the product is processed in plants owned by companies involved in the citrus industry, even though the technology employed is based on that developed for the dairy industry. The products are pasteurized by the HTST (high-temperature short-time) process in which the juice is heated to a high temperature, on the order of 175°-180°F (79.4°-82.2°C) for a very short time, about 0.5 second. It is then cooled and filled into cartons at about 32°-40°F (0°-4.4°C). Such a process is not damaging to the flavor and texture of the juice and the resulting product has a very acceptable flavor and aroma.

Chilled products, which have shelf lives in the order of five to six weeks, are sold at refrigerated temperatures (4.4°-7.2°C) in retail outlets. Open code dating of these products ensures a supply of fresh product for the consumer at retail.

Some chilled products are packaged in glass containers, 32-fl. oz. and 64-fl. oz., but the dairy-type cartons account for the major segment of this market. Berry et al. (11) reported that the quality of these products remained high for long periods of time if maintained at 50°F (10°C) or lower, and products in glass exhibited better ascorbic acid and flavor stability than those in paper or plastic cartons. Higher temperatures led to more rapid deterioration of ascorbic acid and flavor.

Canned and bottled juices are pasteurized at relatively high temperatures (76.7°-90.6°C) and the containers are filled hot. The hot fill serves to sterilize the container and, in the case of a can, it is inverted for 60-90 seconds after seaming to sterilize the lid. The cans are then cooled to about 105°F (40.6°C) in a spin cooler or a spray cooler before being labeled and cased.

Bottles are filled in the same manner, but the caps are sterilized with steam or a chemical sterilant before being applied to the bottle. The filled containers are then cooled gradually in a spray cooler.

When freshly extracted juice is being filled into cans or bottles, the temperature of pasteurization must be sufficient to inactivate the natural enzymes, particularly pectinesterase. For orange juice, this temperature is somewhere between 185° and 195°F (85°-90.6°C) and is, to a degree, dependent upon the pH of the juice. Grapefruit juice, generally, need not be subjected to temperatures as high as are necessary to orange juice in order to achieve stabilization. The temperatures required for grapefruit juice are between 170°-189°F (76.7°-87.2°C). Joslyn and Sedky (7) showed that the heat inactivation of enzymes responsible for cloud instability in grapefruit juice was more rapid at pH 2.5 than at pH 4.0. Rouse and Atkins (9) and Pratt and Powers (12) corroborated these findings. Of course, with any juice the inactivation of enzymes is dependent upon both time and temperature. As the temperature of pasteurization is increased, the length of time in the pasteurizer can be decreased.

1.5.1 Aseptic Packaging

Canned and bottled citrus juices are examples of products that are packed aseptically, and these processes have been used in the industry for many years. One of the newer processes for aseptic packaging employs a paperboard package that is sterilized with hydrogen peroxide prior to the form, fill, and seal operation. This process, developed by Tetra Pak Ab of Lund, Sweden, is in use in many parts of the world, but it has not yet been approved by the U.S. Food and Drug Administration for domestic use.

The new packaging system developed by Tetra Pak is known as the Tetra Brik[®], and is generally available in 1-l, 200-ml, and 250-ml sizes. The system is considered to be an alternative to metal and glass containers. The packaging material comes in roll stock form and is a 6-or 7-layer laminate. Polyethylene and aluminum foil offer the major barrier properties to the package.

The system enables heat sensitive products to be processed with minimal heat input. The processing temperatures employed are similar to those employed for the dairy-type paper cartons. The package integrity, when properly sterilized and maintained, is such that microbial reinfection is inhibited. Because oxygen can permeate the package along the longitudinal and lateral seams, it is not truly hermetic even though it does provide asepsis. One major advantage of the package is that it contains no headspace because the top seal is actually formed through a column of sterile product. This lack of headspace offers some protection, at least initially, to oxygen-sensitive products.

In many of the developing countries of the world, the Tetra Brik[®] system offers the only economical and practical package for juices and juice products (also milk).

The major disadvantages of the Tetra Brik[®] process are the slow line speeds (70 units per minute) and the limited mechanical and physical strength of the package. The latter makes careful handling and adequate secondary packaging quite essential.

2 Nutritional Quality of Citrus Juices

2.1 Vitamin C

Citrus fruits have long been noted as excellent sources of ascorbic acid (Vitamin C), which is the most abundant vitamin in the citrus fruits. Citrus fruits are also quite rich in the mineral element, potassium, and are often recommended for patients who must use diuretic drugs. Healthy adults require 60mg per day of Vitamin C and about 2.5g per day of potassium (13).

Atkins et al. (14) reported that most of the ascorbic acid that occurs in the orange is present as a constituent of the peel. Based on the weight of whole fruit, the juice contains about 25% of the total ascorbic acid content. The juice of the grapefruit contains only about 17% of the total ascorbic acid content on the same basis.

The ascorbic acid content of the juice of different citrus fruits varies considerably, and the content will vary with stage of fruit maturity, fruit variety, and climate. Soil conditions and fertilizing practices have only minimal effects if any at all (15). According to Ting and Attaway (16), oranges generally contain from 40-70mg/100ml of juice, whereas grapefruit, tangerine, and lemon juice contain between 20 and 50mg/100ml of juice.

The ascorbic acid concentration is high in immature fruit, and it decreases as the fruit ripen and increase in size, according to Harding et al. (17).

As with oranges, grapefruit exhibit an inverse relationship between ascorbic acid content and maturity. Metcalfe et al. (18) examined five varieties of grapefruit grown in six locations in the Rio Grand Valley of Texas and concluded that, although there were only small differences among the varieties, there were great variations in ascorbic acid content of the fruit from any given tree. These workers also reported a significant decrease in ascorbic acid content due to maturity. Ross (19) reported variations in ascorbic acid content of grapefruit from trees in different areas, as well as from trees in the same grove. He correlated the ascorbic acid content with acidity and reported that it increased with acidity.

A number of workers examined the effect of light exposure on ascorbic acid content and the general conclusion is that direct sunlight has a positive effect on its content; i.e., exposure to direct sunlight tends to increase the ascorbic acid content of fruit.

Long et al. (20) found that the ascorbic acid content of grapefruit was inversely related to their size. In Valencia oranges, Sites and Reitz (21) found a positive correlation between ascorbic acid and the soluble solids of fruit from the same tree.

As might well be expected, other citrus fruits exhibit the same type of seasonal decline in ascorbic acid content of the juice with maturity. Harding and Sunday (22) reported that the ascorbic acid content of tangerines may be 35mg/100ml of juice in the early season and as low as 10-15mg per 100ml if the fruit is allowed to overmature.

2.2 Other Nutrients of Dietary Significance

Other nutrients in orange juice that are of dietary significance, according to standards set by the U.S. Food and Drug Administration, i.e., they are present at a level of 10% or more of the U.S. RDA (Recommended Daily Allowance) per serving, are folic acid and thiamine (Vitamin B₁). The factor of significance (10% of the U.S. RDA per serving) is set forth in 21CFR 101.9(c) (7)(v)(6). A serving size for orange juice is generally regarded as six fluid ounces or 177ml.

Ting (23) reported values for thiamine in orange juice between 0.75 and 0.85mcg per gram of juice, and a survey of the literature indicates that other citrus juices contain lesser amounts. Analyses of orange concentrate by an industrial laboratory for our Citrus R&D Laboratories resulted in a value of 75mcg per 100g of reconstituted orange juice, in good agreement with the values reported by Ting (23). Adams (24) reports 91mcg/100g for reconstituted orange juice.

In the compilation of Adams (24), reconstituted grapefruit juice is reported to deliver about 38mcg of thiamine per 100g and tangerine juice (reconstituted) about 59mcg/100g. Per serving of 177ml, these values would be 170, 70, and 100mcg, respectively, for orange, grapefruit, and tangerine. With a U.S. RDA of 1.5mg, the percentage of the U.S. RDA of thiamine delivered by these juices would be 11%, 5%, and 7%, respectively.

Folic acid, generically described as folacin, is chemically known as pteroylmonoglutamic acid. There are several compounds that exhibit folic acid activity and they differ only in the number of glutamic acid residues they contain. These polyglutamates, as they are known, must be acted upon by the enzyme, conjugase, to release the folic acid for metabolic activity. A deficiency of this vitamin leads to megaloblastic anemia (25). Most evidence places the daily requirement for folic acid at about 50mcg per day of crystalline folic acid (26,27); however, the U.S. RDA for total food folacin is set at 400mcg for the adolescent, for adult males, and for non-pregnant, non-lactating females. The higher RDA is specified to allow for absorption of only 25% of folic acid activity in a manner comparable to the crystalline folic acid and to allow for a wide range of availability of the polyglutamate form (13).

Early work placed the folacin content of orange juice at between three and six micrograms/100ml (28). Later, Hurdle et al. (29) revised this to 20-45mcg/100g for orange products, and they also reported that canned grapefruit products contained about 11mcg/100g. More recent work by Streiff (30) indicated a folacin value of from 50-100mcg with ingestion of 100-125ml of orange juice. Grapefruit juice and tangerine juice were reported to have lower levels. Dong and Oace (31) reported a folacin value of 50mcg/100ml for orange juice, and a somewhat lower level for grapefruit juice.

Ting et al. (32) reported an average folacin value for reconstituted Florida orange juice of about 45mcg/100ml. In our own studies, with analyses conducted by independent analytical laboratories, we have not seen values of that magnitude, but rather have observed values on the order of 26-33mcg/100ml of juice. These values would be on the order of 12%-15% of the U.S. RDA. Ting (23) has also reported that wide variation in the folic acid content occurs throughout the growing season and that the concentration increases as the season progresses.

2.3 Other Nutrients

A number of nutrients of lesser dietary significance are present in orange juice and other citrus juices. Measurable levels of Vitamin A, riboflavin (Vitamin B₂), niacin, pyridoxine (Vitamin B₆) and pantothenic acid have been reported in orange juice. The levels of these nutrients are generally in the range of 2-3% of their respective U.S. RDA's. For a more extensive review of these nutrients, one should consult Ting (23) and Araujo (33).

In addition to the vitamins mentioned above, citrus juices are a rich source of potassium. Even though potassium is an essential mineral in human nutrition, the U.S. Food and Drug Administration does not include it in its nutritional labeling program because it is widely distributed in foods. The Food and Nutrition Board of the National Academy of Sciences (13) has determined that healthy adults require about 2.5g of potassium per day. Based on the data of McHard et al. (34) a 6-oz. serving (177ml) of orange juice would provide about 0.29g of potassium. Values produced in our own laboratories would approximate a potassium content of about 0.4g per 177ml of orange juice. Ting (23) reports a potassium content of 0.30-0.4g per 177ml for orange juice.

Other mineral elements are present in citrus juices in measurable quantities. McHard et al. (34) reported on the trace element contents of Florida and Brazilian orange juice. They cited concentration ranges for 25 elements. Ting (23) reported that calcium, iron, phosphorus, magnesium, zinc, and copper are present in reconstituted FCQJ at levels equivalent to about 1% to 5% of their respective U.S. RDA's.

Phosphorus reportedly occurs in orange juice at levels of about 10-30mg/100g of juice (24,34,35), equivalent to 1.9-5.6% of the U.S. RDA; magnesium was reported at levels between 8-15mg/100 ml of juice by Birdsall et al. (36), whereas Ingwalson et al. (37) reported levels in reconstituted orange juice of 12-14mg/100g. McHard et al. (34) reported similar values. The maximum would be about 6.5% of the U.S. RDA per 177ml of orange juice.

Orange juice was reported to contain from 50 to 160mcg of copper per 100ml by Birdsall et al. (36), whereas others reported values in the range of 30-50mcg/100g (34,37), a maximum of 1.7% of the U.S. RDA, but possibly as low as 0.3% of the U.S. RDA.

Calcium has been reported at 6.5-15.4mg/100g of reconstituted orange juice (34,35,37). This level, which is 1.2% to 2.7% of the U.S. RDA, is not of any great significance. Likewise, iron, which has been reported at levels of 0.08 to 0.7mg/100g of orange juice (34,37) is not of any great nutritional significance because the level is only 0.8% to 7% of the U.S. RDA.

3 Effects of Processing on Nutritional Quality

Processing as it is practiced in the industry requires the input of heat to effect pasteurization, enzyme stabilization, and/

or concentration. Heat processing to achieve one of these three results would not be expected to have any detrimental effect on the mineral composition of citrus juices. These micronutrients should not be lost during processing; neither should they be degraded. The same cannot be said for the organic micronutrients, the vitamins, and the so-called macronutrients, carbohydrates, proteins, and fats, which supply energy as well as nutrition to the human body.

Citrus fruits are not regarded as good nutritional sources of fat based on 21CFR 101.9(c)(6) that the delivery of less than one gram of fat per serving is not of dietary significance (6). According to Adams (24), reconstituted FCOJ delivers about 0.2g of fat per 177ml; reconstituted frozen concentrated grapefruit juice about 0.2g/177ml. Tangerine juice may be slightly higher in fat content. According to Nagy (38), the lipids that occur in citrus juice contain high unsaturated/saturated fatty acid ratios (> 4).

The contribution of lipid oxidative products to off-flavor development has been studied by many workers, and a review of these studies has been presented by Nagy (38). It is generally agreed that the contribution of the lipid oxidative products to the flavor deterioration of processed citrus products is relatively minor when compared to the contributions by the products formed by the acid-catalyzed hydrolysis of flavoring oils and the products of Maillard browning (39,40).

Citrus juices and their products cannot be considered significant dietary sources of protein because the protein efficiency ratio (PER) of citrus protein is less than 20% that of casein (23, 41).

According to the regulations set forth in 21CFR 101.9(c)(7)(ii)(b), protein with a PER less than 20% that of casein is not of dietary significance (6).

The protein in citrus is generally associated with the solid portions of the fruit, i.e., the seeds, flavedo, albedo, chromatophores, and pulp. Some of these components find their way into the juice along with the available free amino acids during extraction and processing and storage. Studies conducted in our laboratories (42,43,44) and by others (45) have shown that reductions in the pulp content of juice slow the rate of browning.

According to Ting (23,41), a serving (177ml) of reconstituted FCOJ delivers about 19g of carbohydrate and 84 calories contributed primarily by the sugars, sucrose, glucose, and fructose. Adams (24), indicates that a serving of reconstituted FCOJ delivers 92 calories, whereas grapefruit and tangerine juice deliver 76 and 68 calories per 177ml, respectively.

Citrus juices contain both nonreducing (sucrose) and reducing (fructose and glucose) sugars. Mature oranges contain almost equal amounts of the two types and the reducing sugar content is composed of almost equal amounts of fructose and glucose. Grapefruit tend to have nearly equivalent amounts of reducing and non-

reducing sugars, but at times, the reducing sugars tend to be slightly more dominant (16). In tangerines, the nonreducing sugar dominates except in immature fruit; in juice from mature fruit, sucrose may account for 60-65% of the total sugar content. In lemon juice, the reducing sugars dominate (46) and may account for about 90% of the total sugars.

The sugars, which contribute much to the acceptability of citrus juices, under adverse conditions can play a major role in the formation of off flavors that reduce the acceptability of the citrus juices and their products. The sugars, primarily the hexoses, can participate in "browning" reactions that cause darkening of the juice and these reactions give rise to components that are described generally as apricot-like or pineapple-like in flavor. In general, the more processed flavor that a citrus product exhibits, the less acceptable it becomes to the consumer.

Some authors have indicated that the sugar-amino acid reactions of the Maillard type are of minor importance in citrus juices because of the high acidities involved. Studies in our laboratories (42-44) would tend to indicate that, to the contrary, the amino acids and sugars are of more than just minor importance in the darkening of citrus juices. Huffman (42) treated citrus juices with cationic ion-exchange resins to remove amino acids, proteins, and the mineral cations, then restored the cations. The juices from which the free amino acids were removed were less subject to darkening and off-flavor development than were their respective controls after heating for long periods of time to temperatures near 100°C, then storing at room temperature. Juices were also dehydrated in a vacuum shelf dryer and on a chain belt dryer with less visible darkening than control samples. The ion-exchange treated juices were judged by sensory panels to be much more acceptable than untreated controls when presented as pasteurized juice or as dehydrated juice. Additional studies conducted in our laboratories (43) corroborated the findings of Huffman. In addition, lowering the pulp content of juice prior to dehydration decreased the tendency for juice to darken during the drying process. Seaver and Kertesz (47) reported that D-galacturonic and D-glucuronic acids, when heated alone or in the presence of amino acids, formed colored compounds at a rate exceeding that found with common sugars. They further reported that L-ascorbic acid formed colored compounds more rapidly than the sugars, but still at a slower rate than the uronic acids.

Curl (48), in a study conducted with a synthetic orange juice, reported that the loss of ascorbic acid occurred in the presence of citric acid and potassium citrate buffer alone, but that the losses were increased by the addition of the sugars, levulose, sucrose, and dextrose, in that order. He found that darkening of the synthetic juice occurred principally when both amino acids and sugars were present; and, the effect was even more pronounced by the presence of ascorbic acid.

Pruthi and Lal (49), in a study of different methods for

preserving and storing citrus juices, reported that the addition of 5% cane sugar to the juices accelerated the darkening and did not aid in ascorbic acid retention. Kato and Sakurai (50) studied the effects of ascorbic acid, organic acids, amino acids, and inorganic ions on browning in a model system. They determined that 3-deoxyglucosone and 5-hydroxymethylfurfural were formed by the action of organic acid on fructose (formed by inversion of sucrose). Browning, they reported, was affected by organic acids, amino acids, oxidized ascorbic acid, and the inorganic ions, Sn^{+4} , Fe^{+3} , Sn^{+2} , and Al^{+3} . These investigators reported that 3-deoxyglucosone and 5-hydroxymethylfurfural were intermediates in the browning of concentrated lemon juice that occurred when the concentrate was diluted to single-strength juice with a sucrose solution. They concluded that amino acids had a role in the browning (51).

Wolfson et al. (52) studied the nonenzymic browning of dehydrated orange juice and concluded that 4-aminobutyric acid was of particular significance in the formation of colored products. The initial phase of the browning reaction led to a loss of D-glucose and 4-aminobutyric acid. Kampen (53) stored freeze-dried orange juice crystals and a synthetic mixture for 40 days at 50°C and monitored losses of total amino acids (73%), ascorbic acid (100%), citric acid (5.1%), and sucrose (4.4%). The orange juice crystals were discolored from Maillard browning, and several carbonyl compounds and furfural derivatives were identified as products of the reactions. Berry et al. (54) studied foam-mat dried instant orange juice stored at 70°F and at 85°F and reported that the stability of the product was improved by the use of more acidic juices, but adding acid, or removing sugar.

They reported an inverse relationship between stability of the instant orange juice and the pH of the orange concentrate dried.

Although the importance of the sugars in the browning of citrus juices has been debated by many investigators, virtually all agree on the importance of ascorbic acid in this reaction. Joslyn (55) reported that ascorbic acid was the most reactive of the system, ascorbic acid-amino acid-sugar, that occurs in orange juice. Sugars, he reported, exercise a protective effect on the enzymic and nonenzymic oxidation of ascorbic acid; hence, both glucose and fructose are inhibitory to browning. The amino acids were reported to have an inhibitory effect in the early stages of the browning reaction, but in later stages, these components increased the rate of browning.

Moore et al. (56) reported that the decomposition of ascorbic acid in orange juice was directly associated with darkening. According to Curl (57), the development of off flavors in orange juices at 13-71% soluble solids was closely paralleled by the loss of ascorbic acid and by darkening. In mandarin juice, Aiba et al. (58) found that the rate of browning was related to the decomposition of ascorbic acid. Studies with the juice of natsu-

daidai (a Chinese citron) by Imai et al. (59) and others also implicated ascorbic acid as a major reactant responsible for browning. Imai et al. (59) reported that the free amino acids played an important role in the browning of the juice.

Clegg (60) studied the nonenzymic browning of lemon juice and reported that the phenomenon was attributable to ascorbic acid rather than sugar-amino acid condensations. She reported that furfural was produced during the development of browning, but did not consider that it played an important role in the aerobically-produced browning. In model systems that simulated lemon juice, she reported that amino acids in ascorbic systems were major contributors to browning (61).

It has been reported and is generally agreed that ascorbic acid in citrus juices can be degraded through aerobic and anaerobic pathways (53,62). The acid is quite sensitive to oxidation and dehydroascorbic acid is the primary oxidation product, though relatively unstable. It undergoes conversion to 2,3-diketogulonic acid. In addition to these oxidation products, furfural and hydroxyfurfural have been identified as products of the degradation of ascorbic acid (60,63,64,65). Bauernfeind and Pinkert (66) proposed pathways for both the aerobic and anaerobic pathways in aqueous media. In these pathways, shown in Figure 3, furfural can result from either mode of ascorbic acid destruction while hydroxyfurfural is a product of the oxidative system.

In processed citrus products, ascorbic acid loss can occur through aerobic or anaerobic mechanisms. The oxidation of ascorbic acid in orange juice was studied by Evenden and Marsh (67) and was reported to be a first order reaction whose rate was a function of temperature. In a very recent review, Nagy (65) reported that the degradation of ascorbic acid was best explained by a first-order reaction and that for grapefruit juice, the Arrhenius plot showed a linear profile for the temperature region 10–50°C. With orange juice, his data suggested that two different reaction mechanisms were operative with the kinetic change occurring at about 28°C. Between 10° and 27°C the rate of ascorbic acid loss doubled for each 10°C rise; from 27° to 37°C the rate quadrupled. The data of Nagy (65) also confirmed earlier data by Ross (68) and Lamb (69) that indicated for similar storage temperatures the loss of ascorbic acid was greater for orange juice than for grapefruit juice.

4 Processed Citrus Products

Of the 1978–79 domestic citrus crop, some 6,855,000 metric tons of oranges from a total of 8,340,000 metric tons went to the production of processed products. Of the 2,490,000 metric tons of grapefruit that were harvested, 1,510,000 metric tons were utilized in processed products. A similar picture can be painted for the other domestic citrus crops. It is easy to see that the market for processed citrus fruit in the U.S. is ex-

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In Citrus Nutrition and Quality; Nagy, S., et al.;

ACS Symposium Series; American Chemical Society: Washington, DC, 1980.

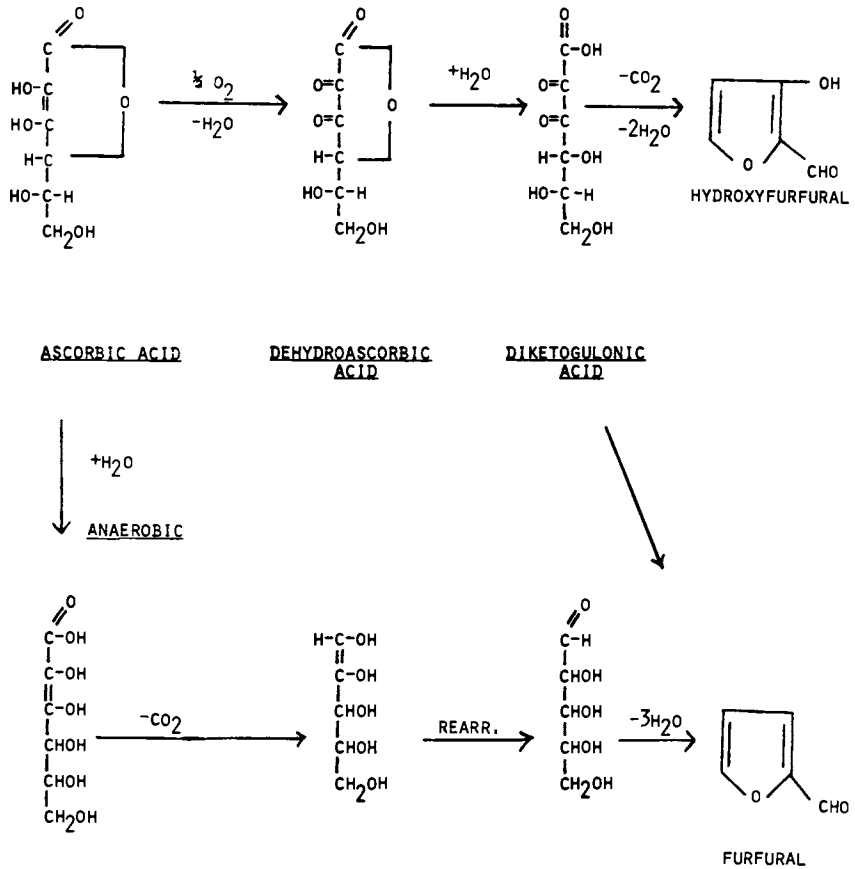


Figure 3. Possible ascorbic acid degradation pathways

tremely important to the citrus industry. Almost 95% of the Florida orange crop is utilized by processors in the production of juice products. Since the diets of domestic consumers contain processed products as their major source of citrus, it is reasonable to look at these products and how they are affected nutritionally by the processing techniques in practice in the industry.

4.1 Frozen Concentrated Juices

Frozen concentrated orange juice (FCOJ) is by far the most widely distributed of the processed citrus products. First marketed in the mid-1940's, it has grown in consumer acceptance until the present day, and to the point where its volume consumption exceeds the combined total for all other processed citrus products.

FCOJ and other frozen concentrated citrus juices are produced by the process outlined in Figure 4. Prior to evaporation the process includes extraction, finishing, and blending. In the evaporator, the juice may be concentrated to 45°Brix (% soluble solids) or higher; and, as a matter of routine practice, most of the evaporator pumpout (concentrate) is at 65-68°Brix. The concentrate can go to low-temperature storage or directly to processing for FCOJ. During the fruit processing season, cut-back juice may be used to dilute the concentrate to 45°Brix. At other times, essence and water are used to prepare FCOJ. Berry and Veldhuis (3) reviewed this process in great detail.

The loss of ascorbic acid during extraction, finishing, and blending is minimal. Based on the data of Sale (70) and Hayes et al. (71), the loss should be no greater than 2%. Hayes et al. (71) prepared orange juice concentrates having 50-60% solids and reported a mean retention of 96.6% of ascorbic acid.

Ting et al. (32) collected samples of FCOJ from 23 manufacturing plants in Florida during 1973 and 1974 and analyzed them for selected nutrients, those specified by the U.S. Food and Drug Administration as being essential to human nutrition. The average nutrient content of FCOJ reconstituted to 12.8°Brix expressed as percent of the U.S. RDA is shown in Table VII along with the U.S. RDA's as specified by the Food and Drug Administration (6). Based on these data, it can be seen that FCOJ is of dietary significance with respect to Vitamin C (ascorbic acid), folic acid, and thiamine, i.e., it provides 10% or more of the respective U.S. RDA per 177ml serving.

Much has been written about the effects of processing, temperature, and storage conditions on the stability of ascorbic acid in citrus juices. On the other hand, little is known about the stability of folic acid under similar conditions. Chen and Cooper (72) studied the effects of temperature and oxygen and ascorbic acid on the thermal degradation of folic acid, and they reported that ascorbic acid increased the stability of the tetra-

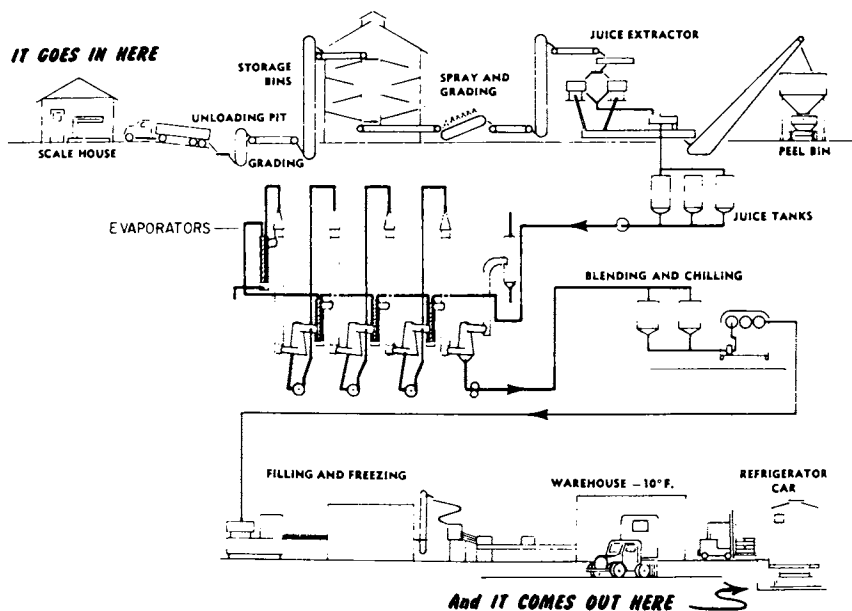


Figure 4. Flow diagram for frozen concentrated orange juice production (courtesy Adney Reed)

TABLE VII

Average Nutrient Delivery per Serving of Reconstituted
FCOJ (12.8°Brix) in Relation to U.S. RDA

Nutrient	U.S. RDA (1)	Average % U.S. RDA/ 177ml FCOJ(2)
Vitamin A	5000 IU	1.4
Vitamin C	60 mg	131
Thiamine	1.5mg	9.8
Riboflavin	1.7mg	2.4
Niacin	20 mg	2.0
Calcium	1.0g	1.8
Iron	18 mg	1.1
Vitamin B ₆	2 mg	4.9
Folic Acid	0.4mg	20.3
Phosphorus	1.0g	3.3
Magnesium	400 mg	4.9
Zinc	15 mg	0.7
Copper	2 mg	4.4
Pantothenic Acid	10 mg	3.3
Vitamin D	400 IU	---
Vitamin E	30 IU	---
Vitamin B ₁₂	6 mcg	---
Iodine	150 mcg	---
Biotin	0.3 mg	---

Source: 1) U.S. Food and Drug Administration (6).
2) Ting et al. (32).

hydro- and 5-methylfolic acid at 100°C. Their data indicated that the degradation of these folates at high temperature was due to an oxidative process that required the presence of molecular oxygen.

Floyd and Rogers (73) analyzed authentic samples of Florida orange juices and concentrates to determine the effects of concentration on chemical composition. They reported no significant effects of concentration on the chemical composition of orange juice concentrate as compared to single-strength juice. Setty et al. (74) reported that the nutrient content of mandarin orange juice when concentrated to 62°Brix and higher was very little affected.

Horton and Dickman (75) reported that the physiologically available ascorbic acid (ascorbic acid and dehydroascorbic acid) in reconstituted orange juice was stable over a two-week period, both at 4°C and at room temperature. Aeration caused by blendorizing at high speed for two minutes had no effect on ascorbic acid stability.

Bissett and Berry (76) reported on the ascorbic acid retention in orange juice as a function of container type. They stored FCOJ in foil-lined cardboard, rectangular cartons and in polyethylene (PE)-lined fiber cylindrical cans for a year at -20.5°, -6.7°, and 1.1°C. At -20.5°C, the ascorbic acid retention was 93.5% in the foil-lined cartons and 91.5% in the PE-lined cans. Neither container proved effective above freezing due to microbial spoilage. The foil-lined carton was superior at 1.1°C, in that 89% of the ascorbic acid was retained after three months. In the PE-lined can, the retention was 44% after three months at 1.1°C.

Garcia (77) studied the effect of storage on 45°Brix and 54°Brix orange juice concentrates packaged in 6-oz. foil-lined, spiral-wound cans and in 200-ml foil-lined, rectangular cartons (Tetra Brik®). The latter were both cold filled and aseptically filled via high-temperature short-time pasteurization. These products were stored for one year at -17.8°, 7.2°, and 23.9°C. The aseptically-processed concentrates retained sterility throughout the course of the study and were stored at 7.2° and 23.9°C. The samples in 6-oz. foil-lined composite cans and those cold filled into the 200-ml rectangular packages were stored only at -17.8°C because they were not aseptically packed and were subject to microbial spoilage.

Figures 5 and 6 show the ascorbic acid retention in the 200-ml packages as a function of time. The data for the 6-oz. composite can are not shown because the ascorbic acid retention in these packages was similar to but just marginally poorer than was the retention in the 200-ml packages at -17.8°C. This marginal difference was attributed to the presence of some head-space oxygen in the 6-oz. cans which resulted in the loss of slightly more ascorbic acid.

At -17.8°C, the retention of ascorbic acid over 12 months

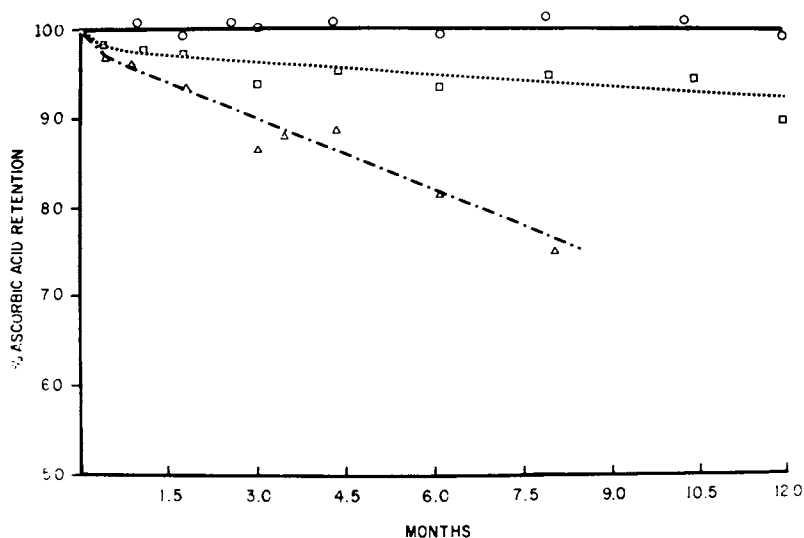


Figure 5. Ascorbic acid retention in 54° Brix concentrated orange juice as a function of storage temperature ((\circ) -17.8°C; (\square) 7.2°C; (\triangle) 23.9°C)

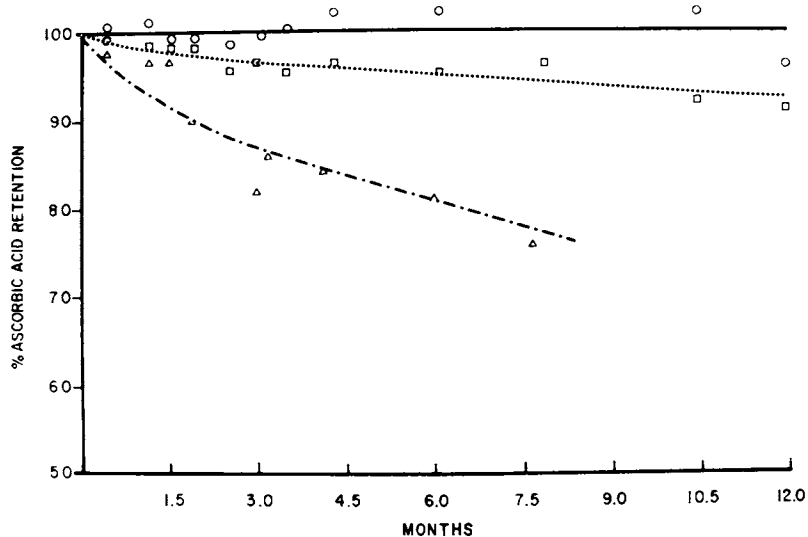


Figure 6. Ascorbic acid retention in 45° Brix concentrated orange juice as a function of storage temperature ((\circ) -17.8°C; (\square) 7.2°C; (\triangle) 23.9°C)

exceeded 97% in the 200-ml packages and 94% in the 6-oz. composite cans.

The ascorbic acid retention in both the 45°Brix and 54°Brix concentrates stored at 7.2°C were very similar over 12 months. The loss of ascorbic acid amounted to about 8% to 10% over the storage period.

At 23.9°C, the 45°Brix concentrate initially exhibited a more rapid loss of ascorbic acid than did the 54°Brix concentrate, but at the end of eight months both had lost about 25% of their starting ascorbic acid. The more rapid loss of ascorbic acid may have been caused by the amount of dissolved oxygen in the 45°Brix concentrate initially. It may have been the result of more rapid diffusion of oxygen into the lower °Brix concentrate once it entered the package along the longitudinal seam and at the corners. Neither factor was measured.

As might have been expected, flavor deterioration followed the same pattern as did the degradation of ascorbic acid, i.e., the concentrates at 23.9°C deteriorated in flavor acceptability more rapidly than did those at 7.2°C; those at 7.2°C deteriorated at a faster rate than did those stored frozen. The products stored at 23.9°C remained acceptable in flavor for about six months; those at 7.2°C remained acceptable for eight to ten months. At -17.8°C, the products were still acceptable after a year of storage.

Packages of the type used in this study by Garcia are presently used in many parts of the world for the packaging of milk, juices, and juice products. Brik Pak, Inc., a subsidiary of Tetra Pak Ab has petitioned the Food and Drug Administration for approval to use the Tetra Brik® package in the U.S., but a final decision regarding their petition is still pending.

Frozen concentrated grapefruit juice is produced in essentially the same manner as FCOJ. Prior to evaporation, the juice is stabilized at 66° to 88°C to prevent gelation and clarification during storage. As is done in the manufacture of FCOJ, coldpressed grapefruit peel oil is added to the grapefruit concentrate to enhance its flavor. Essence, the volatile water soluble flavor from the fruit, may be added during the manufacture of a grapefruit concentrate, but this system of flavors does not seem essential to high flavor quality. This is contrary to what is generally found with FCOJ where essences do enhance flavor quality (78).

4.2 Reduced-Acid Frozen Concentrated Orange Juice

Interest in reduced-acid citrus juices originated in the early 1960's when Kilburn and Drager (4) employed electrodialysis to remove citrate ions from juice. The Florida Department of Citrus tested the reduced-acid concept with consumers at the New York World's Fair in 1965, and followed this test with a national consumer survey in 1972. The Coca-Cola Company Foods Division

conducted an independent consumer survey in 1973, and all of the studies indicated substantial consumer interest in reduced-acid citrus juices. After further testing with consumers in which products were tested in homes, the Foods Division was granted a permit from the U.S. Food and Drug Administration and the Florida Department of Citrus to manufacture the product and to distribute it in interstate commerce. Later the FDA was petitioned to establish a new standard of identity for reduced-acid frozen concentrated orange juice. The issuance of that standard is still pending.

A reduced-acid frozen concentrated orange juice is presently being test marketed by The Coca-Cola Company Foods Division. This product is produced by blending regular concentrated orange juice with acid-reduced concentrate in proportions that will result in a final frozen concentrated orange juice with a Brix/acid ratio between 21 and 26 to 1; the precise blend being dependent on the Brix/acid ratios of the two concentrates employed. After blending, the concentrate is adjusted to 45°Brix through the addition of water and essence. Coldpressed orange oil is added for good flavor quality. The product is then canned and stored at -17.8°C.

The fates of the nutrients of orange juice were naturally of concern in the process of producing an acid-reduced orange concentrate, so many studies were conducted to ascertain the levels of the major nutrients before and after processing. The major concerns were in regard to ascorbic and folic acids, since these components might well be removed during the ion-exchange process to remove citrate ion.

Since the anionic resin employed is weakly basic, the retention of stronger acids is favored with respect to the weaker acids, ascorbic and folic. Also, because of the law of mass action, the removal of citrate ion is favored over ascorbate and folate.

The change in the ascorbic acid concentration of juice during acid reduction processing is illustrated in Figure 7. Some ascorbic acid is initially retained by the resin but it is later replaced by the stronger acid, citric, as the exchange capacity of the resin is depleted. This initial reduction in ascorbic acid is on the order of 15%, but as the column is exhausted this acid is replaced by citric and it is eluted in the juice stream. Near the end of treatment, the ascorbic acid level rises to its initial level and even exceeds it as that which was initially held by the column is replaced by citrate.

It can be seen in Figure 7 that some ascorbic acid can be lost if the ion-exchange resin is not completely exhausted during processing. When the column is exhausted this loss of ascorbic acid is minimized.

Ascorbic acid loss in acid-reduced juices never exceeded 10% except in cases where the ion-exchange resin was not completely exhausted and generally it was in the range of 3% to

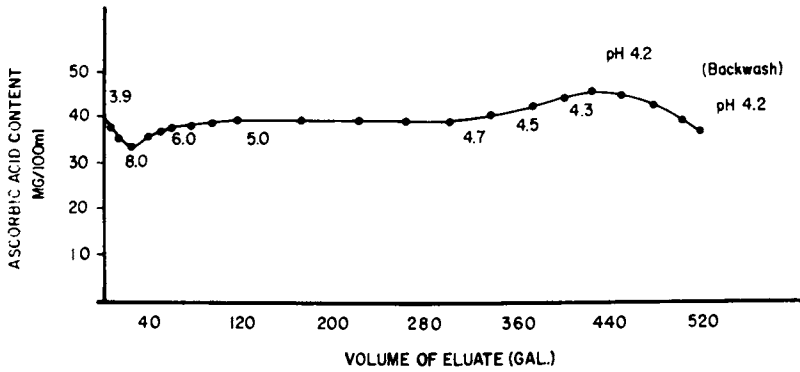


Figure 7. Change in ascorbic acid level during acid reduction by ion exchange

7%. When the acid-reduced concentrates are blended with regular (untreated) orange concentrates to produce a retail product, the reduction in ascorbic acid levels should not exceed 3%; hence, the reduced-acid FCOJ will have an ascorbic acid level within the range of acceptable levels of regular FCOJ. The changes in ascorbic acid levels during acid reduction are generally no greater than those that might be experienced in normal juice and concentrate processing.

The analysis of folic acid is accomplished by a microbiological assay employing either one of two organisms, *L. casei* or *S. faecalis*. The latter organism reportedly gives more reproducible results but the former gives greater sensitivity. Generally, for normal levels of folate in orange juice, the *L. casei* method is the one of choice; however, its reproducibility is probably only on the order of $\pm 20\%$.

Our Citrus R&D Laboratories are not equipped for the routine analysis of folic acid so samples of acid-reduced orange concentrate were submitted to two independent laboratories, and they were also analyzed at the Citrus Experiment Station in Lake Alfred, Florida. Typically, samples of freshly extracted orange juice or orange juice concentrate diluted to 12.8°Brix were found to contain between 21 and 30 micrograms (mcg) of folic acid per 100ml of juice. As a general rule, acid-reduced orange juice, after pH adjustment, going to the TASTE evaporator contained folic acid levels equivalent to the starting juice or concentrate. Some typical analyses are shown in Table VIII.

The total nitrogen content of acid-reduced orange juice was not different from that of regular orange juice, but the free amino acids, as determined by formol titrations, were 3% to 4% higher in the acid-reduced juice. This slight increase in free amino acids may have resulted from some protein hydrolysis during the ion-exchange process.

Other nutrients were measured in acid-reduced orange juice and, for the most part, no significant changes were observed from what would be expected for freshly extracted juice or reconstituted FCOJ. Some data relative to the other nutrients are presented in Tables IX and X.

Based on a review of all the data gathered for reduced-acid FCOJ, it is apparent that any changes that do occur are of an insignificant nature and do not alter the nutritional quality of the processed orange juice. The product of commerce is of equivalent quality to the more popular product, frozen concentrated orange juice.

4.3 Chilled Citrus Juices

The market for chilled citrus juices is one of the fastest growing segments of the domestic retail market, and now is second only to FCOJ in terms of volume consumption. Since its inception in the mid-fifties, this category for processed citrus juices has

TABLE VIII

Folic Acid Content of Acid-Reduced Orange Juice

Description	Folic Acid Content (mcg/100ml)
Diluted Orange Juice Concentrate (12.8°Brix)	
Before Centrifuge (Pulp Removal)	26.3
After Centrifuge	30.7
IE Column Eluate (Juice)	21.4
After pH Adjustment*	32.9
Fresh Orange Juice	
Before Centrifuge (Pulp Removal)	21.6
After Centrifuge	21.3
IE Column Eluate (Juice)	16.5
After pH Adjustment*	22.0

*pH Adjusted to 4.5 by Addition of Juice and Pulp Removed by Centrifugation

TABLE IX

Nutrient Content of Acid-Reduced Orange Juice

Nutrient	Nutrient Content/100ml of 12.8°Brix	
	Before I-E Column	After I-E Column
Vitamin B ₁ (Thiamine)	0.14 mg	0.13 mg
Vitamin B ₂ (Riboflavin)	0.085mg	0.072mg
Vitamin B ₆ (Pyridoxine)	0.080mg	0.086mg
Niacin	0.68 mg	0.69 mg
Pantothenic Acid	0.29 mg	0.44 mg

TABLE X

Range of Mineral Content of Acid-Reduced Orange Juice

Mineral	Mineral Content (ppm)	
	Reported in Orange Juice ⁽¹⁾	After I-E Column
Sodium	2- 24	7- 35
Potassium	1500-2300	1945-2346
Calcium	50- 150	66- 144
Magnesium	90- 130	115- 138
Iron	0.2- 5.0	0.3- 1.4
Copper	0.25-0.33	0.3- 0.4

(1) As compiled from the literature.

experienced some major changes in packaging, and its future probably holds much of the same.

Chilled juices being marketed domestically are prepared either from freshly extracted juice or from concentrate that has been bulk shipped to a packaging site then reconstituted to single-strength juice.

Early chilled juice products were packaged in paper milk cartons and in polyethylene containers. These containers have largely been supplanted by glass bottles and more recently by polyethylene-lined paper cartons. In some cases, the cartons also contain a layer of aluminum foil between the paper and polyethylene. These containers are not hermetic and in many cases are not sterile; thus, even though the juice is pasteurized, the final product is perishable. It may become recontaminated by the package, although this does not often occur. The major problem encountered with these cartons is that they permit transfer of oxygen into the product with the resultant flavor and ascorbic acid degradation; hence, they have relatively short shelf lives. Open code dating, in common practice in the food industry today for perishable products, indicates that the shelf life of a citrus juice in a dairy-type container is in the order of 28 to 42 days depending on the juice and the construction of the juice board in the package. These products must be distributed through channels that allow for refrigeration. Primary storage (i.e., after packaging and before being delivered to the retail outlet) temperatures near 0°C are often specified.

Juices in glass bottles are considered commercially sterile

because the bottles and caps are sterilized and the juice is pasteurized then cooled before the containers are filled. Since there is no oxygen transfer that can occur, more prolonged shelf stabilities are generally realized. These products, when distributed through refrigerated channels, will maintain good flavor acceptability for periods up to ten months. Ascorbic acid stability through this period is also good. At times, such products are distributed at ambient temperature; hence, the shelf life as measured by flavor acceptability and ascorbic acid stability is shortened considerably, perhaps by as much as four to five months.

Berry et al. (11) and Bissett and Berry (76) stored single-strength orange juice (SSOJ) in glass, polyethylene, and polystyrene bottles and in wax-coated cardboard cartons at several temperatures for various periods of time. They concluded that chilled SSOJ in glass containers retained a high level of ascorbic acid for 32 weeks when stored at temperatures not exceeding 10°C. Juices stored in the plastic bottles and in the wax-lined cartons lost 80% of their ascorbic acid in three to four weeks. They concluded that the acceptable shelf life for juice in polystyrene bottles and in waxed cartons was two to three weeks at normal refrigeration temperatures.

Squires and Hanna (79) examined 17 brands of reconstituted orange juice in plastic-coated cardboard containers that were purchased at the retail level then stored at 4°C. Their data indicated that the ascorbic acid contents of these chilled orange juice samples decreased at a rate of about 2% per day. They concluded that the acceptable shelf life for these products was 20 days, at which time the ascorbic acid retention had decreased by about 39% to a level of 28mg/100ml of juice (49.6mg/177ml serving or 82.6% of the U.S. RDA).

Our Technical Department (80) conducted a study in which 80 retail samples of orange juice from concentrate packaged in 32-oz. and 64-oz. plastic-lined cartons were picked up from 14 different retail outlets. The mean age of all samples was 20.2 days; 22.3 days for samples in 32-oz. containers and 18.5 days for samples in 64-oz. containers. The ascorbic acid content of each sample was determined and the average for all samples was 35.2mg/100ml (62.5mg/177ml or 105% of the U.S. RDA per serving). The samples in 32-oz. cartons averaged 33.1mg of ascorbic acid/100ml (58.7mg/177ml or 98% of the U.S. RDA); the samples in 64-oz. cartons averaged 37.7mg/100ml (66.8mg/177ml or 111% of the U.S. RDA). Of all samples analyzed, 53% delivered at least 100% of the U.S. RDA of ascorbic acid per 177ml serving, and all delivered at least 75% of the U.S. RDA of ascorbic acid per serving.

These data would suggest that, although there is some loss of ascorbic acid during the normal shelf life of orange juice in plastic-coated paper cartons, this processed product represents a significant source of ascorbic acid in the diet of any consumer at any time during its dated life expectancy (as indicated by the

date code on the carton). Ninety-six percent of the samples delivered at least 28mg of ascorbic acid per 100ml of juice, the criterion set forth by Squires and Hanna (79).

Paper cartons with an aluminum-foil barrier between the paper and plastic are used for the distribution of some chilled products. Because of the foil barrier, products in these cartons exhibit better ascorbic acid retention than do the plastic-lined cartons. As an example, grapefruit juice stored in such cartons retained 74% of its ascorbic acid over a 40-day storage period at 4.4°C. The rate of ascorbic acid loss was 0.7% per day. Grapefruit juice in a plastic-lined container lost 1.5% of its ascorbic acid per day when stored under similar conditions.

4.4 Canned Juices

Canned grapefruit juice still represents the major segment of the market for processed grapefruit, as can be seen in Table VI. Canned orange juice, on the other hand, represents only a very small percentage of the orange crop that is processed, and its share of the total market for processed oranges is still on the decline. The greater acceptability of FCOJ and chilled orange juice and the increasing growth of the chilled orange juice market segment are the major reasons for the declining market for canned orange juice.

In the infancy of the market for processed citrus juices, the canned single-strength juices were the first products to be widely distributed. In today's market, the main strengths of canned orange juice are its perceived convenience, economy, and ease of storage. Chilled orange juice, on the other hand, is generally regarded by the consumer as being more like fresh orange juice.

Canned orange juice is normally prepared from freshly extracted juice; however, that may not be the case with grapefruit juice. Since the growth in popularity of bottled grapefruit juice, a sizeable portion of that market consists of grapefruit juice from concentrate, as is also the case with chilled grapefruit juice.

In the preparation of canned orange juice, the juice is extracted from fruit, finished and blended to achieve the best possible physical characteristics of the juice, i.e., color, cloud, sweetness, and acidity. After blending the juice is deaerated, deoiled, pasteurized, and canned.

Deaeration is utilized to remove dissolved oxygen from the juice, thereby retarding the oxidation of ascorbic acid and flavor changes that may result from a combination of oxidative browning and oxidative changes in the flavoring oils (81). Deoiling can be accomplished by flash evaporation under vacuum (82), after which the oil content can be readjusted to achieve an acceptable level, which should not exceed 0.035% v/v for grade A orange juice and is generally below 0.020% v/v. For grapefruit juice,

the level is usually lower than 0.015% v/v.

Pasteurization and stabilization of orange juice are generally accomplished by heating to 88-93°C for up to 40 seconds. Grapefruit juice can be pasteurized and stabilized at lower temperatures, perhaps 71-82°C, because of its higher acidity. Following pasteurization the juice is filled into cans or bottles.

Flavor changes that occur in citrus juices are the result of heat input into the product over time; i.e., they are a function of temperature and time. It is for this reason that canned and bottled juices are generally less preferred by consumers than other processed citrus juices, e.g., frozen concentrates or chilled juices. The canned juices receive more heat input during pasteurization and they remain at relatively high temperatures for extended periods of time because they are discharged from the water coolers at temperatures near 40°C to facilitate drying and to inhibit rusting of the cans. It is well known that the rate of flavor deterioration increases with temperature, so canned juices are stored at a temperature as low as is economically practical before distribution at the retail level to extend their shelf life as much as possible.

Ross (68) studied the flavor deterioration and ascorbic acid retention in canned juices, and he concluded that storage temperature was very important to ascorbic acid retention. Further, he found that flavor acceptability was dependent on both time and temperature of storage and that it roughly paralleled ascorbic acid retention. Between 10° and 27°C, the rate of ascorbic acid degradation doubled for a 10° rise in temperature; between 27° and 37°C the rate quadrupled. Studies by Nagy and Smoot (83) and Nagy (65) corroborated these results and further confirmed the finding that ascorbic acid loss is greater in orange juice than in grapefruit juice when stored at similar temperatures.

Moore (84) reported that the ascorbic acid retention was 89.2% for bottled orange juice and 93.2% for canned orange juice stored at 4.4°C for 18 months. At 24.4°C, the ascorbic acid retentions after 18 months were 50.9% and 59.8% for bottled and canned orange juice, respectively. This effect of container type was further demonstrated in studies by Riester et al. (85), Curl (48), and by Moore et al. (86). These studies showed greater losses of ascorbic acid in enamel-lined cans than in plain tin cans. The difference was attributed to the protective effect of the tin, in that oxygen reacted with tin in one case and with ascorbic acid in the other.

A composite, flexible package in broad use throughout much of the world today is the aseptic Tetra Brik[®], a rectangular package of laminate construction containing six or seven components with paper as the primary one. Juices are distributed and retailed in parts of Europe, Asia, South and North America in 200- and 250-ml and in 1-liter Tetra Brik[®] packages, normally at ambient temperature.

Studies were conducted in our laboratories in which single-strength orange juice from concentrate was aseptically packed into liter Tetra Brik[®] packages and also was hot-filled into 1.36-l (46-oz.) enamel-lined cans with plain tin ends. After packing, the products were stored for 18 weeks at 23.9°F and the rate of ascorbic acid retention and flavor deterioration were measured. The results for the ascorbic acid retention are shown in Figure 8. In the metal can there was a rapid initial loss of ascorbic acid due to the dissolved oxygen and headspace oxygen. After this initial loss, the ascorbic acid level stabilized at about 82% of its initial level. In the Tetra Brik[®], a rapid initial loss of ascorbic acid occurred, likely attributable to the dissolved oxygen since there is no headspace in the package. The product in the Tetra Brik[®] package continued to lose ascorbic acid at a linear rate between 6 and 18 weeks until the study was terminated because the product reached a point of borderline acceptability. At that point, the ascorbic acid retention was about 69%. The data from this study would tend to indicate that the shelf-life expectancy for a citrus product in the Tetra Brik[®] package should not exceed five months, and perhaps four months would be a more prudent figure. The attainable shelf life will, of course, be dependent upon conditions under which the product is stored. At temperatures above 23.9°C a shorter shelf life should be expected.

4.5 Dehydrated Juices

Attempts to prepare dehydrated citrus juices date back to the mid-1940's when freeze drying was investigated, followed by an investigation into puff drying in a vacuum shelf dryer (87). Interest in the production of dehydrated citrus juices later led to the development of the continuous vacuum belt dryer and the foam-mat process. Other attempts to dehydrate citrus juices employed drum drying and spray drying. More recently, a filtermat process (88) was described and touted as a method suitable for the dehydration of sensitive products such as citrus juices. The filtermat dryer is described as a four-stage process that utilizes a combination of spray drying and belt drying with heated air; the latter is accomplished on a stainless-steel screen mesh conveyor. Attiyate (89) recently described the process that utilizes microwave energy for evaporation of moisture. The process is said to rank between spray drying and freeze drying from an economic standpoint. A commercial operation exists in France where pre-concentrated orange juice with a total solids content of 63% is dehydrated. The process is said to provide a quality product that exhibits good retention of color, flavor, and aroma.

In most processes for the production of dehydrated citrus juices, a concentrated juice is blended with a drying aid which

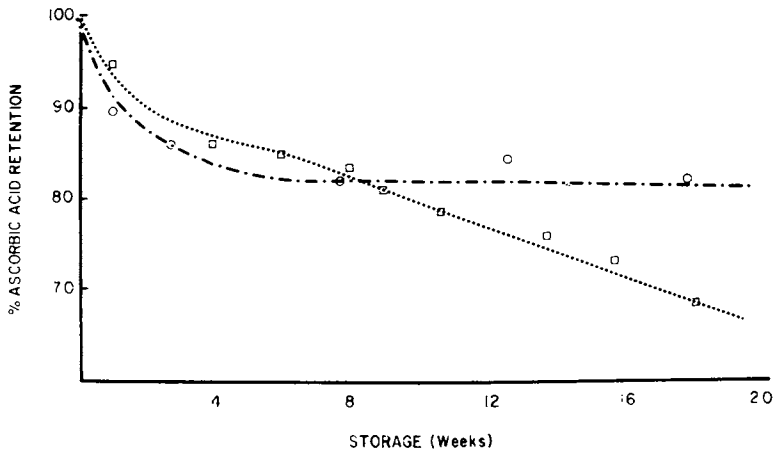


Figure 8. Ascorbic acid retention in single-strength orange juice stored at 23.9°C ((○) 1.36-l metal can; (□) 1.0-l foil-lined Brik (aseptic))

is usually a carbohydrate. The drying aid is generally added in order to improve the drying rate, to inhibit product from sticking to the walls and belts of dryers by raising the "sticky point," to reduce hygroscopicity, and to maintain flowability in the dry powder. The drying agents normally employed are the maltodextrins; however, sugar, low D.E. corn syrups, modified food starches, and other carbohydrates of high molecular weight are also used at times. For foam-mat drying, methylcellulose is used to help create a stable foam and to create a porous structure to enhance the rate of drying.

Vacuum belt drying and spray drying are the two processes most widely used today for the dehydration of citrus juices. In neither process is it commercially feasible to produce a dehydrated juice without the addition of a drying aid, although 100% orange juice has been produced with the continuous vacuum belt dehydrator (42,43). This product is extremely hygroscopic and very temperature sensitive. As a result, the product "cakes" or hardens if exposed to moist air or to temperatures much above 24°C. The product is also subject to browning if not stored at refrigerated temperatures. Dehydrated citrus juices are produced on a vacuum belt dryer at Crystals International, Plant City, Florida, and are items of commerce.

Moy and Speilmann (90) recently reported on the economic feasibility of vacuum puff freeze drying of tropical fruit juices and nectars. They considered the process economically feasible if production rates were 250,000 or 1,000,000kg of dried nectar base per year (two plant sizes) with an assumed level of 35% sucrose (wet weight basis) blended with the juice or puree before dehydration. One assumption made in their study was that a marketing share equivalent to 0.5% of the annual orange juice volume in the U.S. was attainable.

Gupta (90) developed a process for spray drying an aqueous orange juice slurry that contained a carbohydrate drying aid. With his process, he produced free-flowing orange juice powders that contained as much as 60% orange juice solids by weight. There are citrus juice powders commercially available today that contain as much as 50% w/w fruit solids, the remainder of the dry weight being contributed by carbohydrate drying aid.

Because of the need to employ drying aids, the economics for most dehydrated citrus juices are not favorable enough to warrant their large scale usage. Generally, they are employed in specialized areas or in products that contain juice contents in the range of 10-15% by volume of reconstituted product. The flavor qualities of the dehydrated juice products are not equivalent to those of the juices prior to dehydration. Usually the flavor quality is degraded to some degree and this is dependent upon the conditions employed in the drying process (3). If the drying aids are not carefully selected, they can lead to the development of additional off flavors during the dehydration process and the storage of the free-flowing powder.

Berry et al. (54) evaluated foam-mat dried instant orange juice and determined that its flavor was acceptable over 26 weeks when stored at 21.1°C. At 29.4°C flavor changes were observed after two to four weeks with samples in the pH range of 4 to 6. The flavor stability of the instant orange powder was directly related to pH when stored at 29.4°C. Stability was improved by using more acidic juices, addition of acid, or removal of sugar.

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Quality of Citrus Specialty Products

Dried Pulp, Peel Oils, Pulp-Wash Solids, Dried Juice Sacs

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Expansion of processed citrus products into world markets has increased production and processing of citrus in those areas of the world where the climate is suitable for the growth of the crop. Large scale manufacturing of juices and concentrate creates waste and necessitates utilization of the remaining portions of the fruit as by-products or specialty products. Juice and concentrate quality is controlled by regulations, resulting in manufacture to uniform standards worldwide; however, less control is exercised over certain by-products and specialty products. Some of the factors important to quality of the specialty products, dried pulp, peel oils, pulp-wash solids, and dried juice sacs will be included in the following discussion.

Dried Pulp and Pellets

Data from a statistical report (1) indicated that over one million tons of dried citrus pulp and pellets were produced during a recent season from the Florida crop, which was about 70% of total U. S. citrus production. This by-product is important to the function of the citrus processing industry and to many livestock producers who use it as a cattle feed supplement.

The peel, internal membranes, ruptured juice vesicles and seed residue remaining after juice extraction represent the raw material for production of dried citrus pulp. This residue, in its wet state, contains 75-85% water and ferments or sours readily because of the presence of soluble sugars. The difficulty of handling this wet material necessitates dehydration to a moisture content in the range of 10% water. Once dried, if proper precautions are taken to maintain dry conditions, the product may be handled, stored, and shipped in a manner similar to other dry feed stuffs.

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Particle size. Uniform particle size is important during dehydration. If large pieces are present, drying conditions must be altered to include longer drying times; since fine particles and dust which dry rapidly may burn and contribute to product losses and air pollution problems.

Figure 1 illustrates a typical particle size distribution of some feed mill pulp fractions. The distribution of wet press cake and dried pulp fractions were similar in that a fraction of the particles were about 1 mm with another fraction distributed in the 2 to 5 mm range (2). The major particle distributions of the meal and dust portions of the fines were below 1 mm.

Moisture content. The importance of maintaining a low moisture content for either dried loose pulp or pellets has been documented (3). Fire or smouldering is a distinct danger in feed mill operations or storage facilities and usually occurs when the moisture content of the dried products exceeds the recommended 10% moisture level.

Moisture equilibrium during storage of loose, dried citrus pulp at 60% relative humidity (RH), 26°C, has been shown to occur at about 11 to 12% moisture (4). Other researchers have shown this equilibrium to take from 2 to 3 weeks (5). For samples of loose commercial pulp, it was shown that above 75% RH, at 25°C, moisture equilibrium occurred in about 2 weeks (2). However, less time was required at lower relative humidities (30 and 50%), with equilibrium moistures near the initial moisture content of the sample (8.8%).

Moisture equilibrium of dried pellets takes longer to occur than for loose pulp. Results shown in Figure 2 indicate that above 75% RH at 25°C, equilibrium had not occurred within 28 days. The samples at 31 and 52% RH reached equilibrium within one week. Mold growth in the pellets at 90 and 100% RH atmospheres commenced after 28 and 25 days, respectively. No mold growth occurred within 28 days in the other samples. In order to maintain the moisture content of dried pulp or pellets in the range of 10 to 12%, contact with air of greater than 50 to 60% RH at 25°C should be minimized.

Other properties. Some properties of two common-sized commercial pellets are included in Table I. The bulk density of pellets is approximately twice the value for loose pulp. The % void space reflects the compaction effect occurring as a result of pelletizing. Savings in costs for handling and shipping occur as a result of manufacturing pellets because of a large decrease in volume. There is also some space advantage in making smaller diameter pellets with greater bulk density.

Nutrient composition. The nutritional quality of dried citrus pulp may be affected by processing conditions, particularly dehydration temperatures. Pulp dried with dryer exit-stack gas temperatures greater than 143°C shows caramelization or browning. Ammerman et al. (6) and Chapman et al. (7) have shown that a

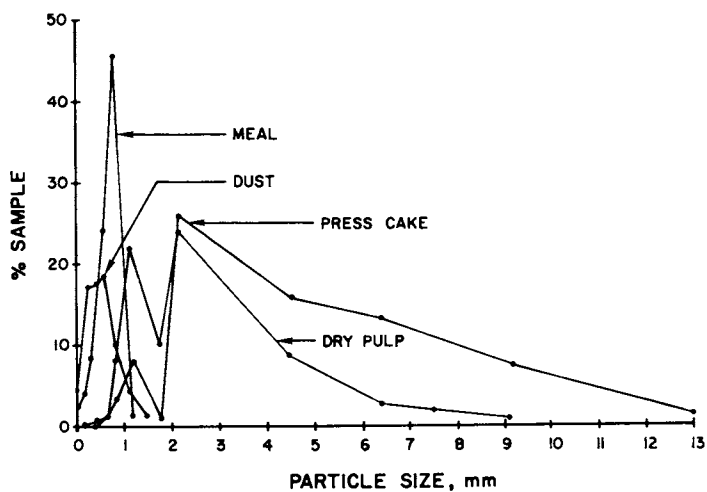


Figure 1. Particle size distribution of citrus press cake, dried pulp, meal, and dust (2)

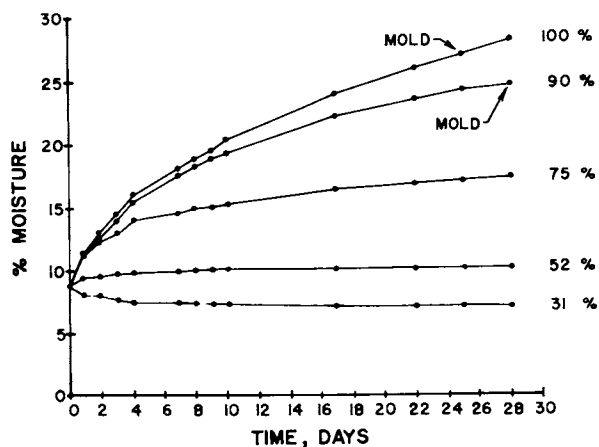


Figure 2. Moisture equilibrium of citrus pellets at 25°C and 31, 52, 75, 90, and 100% relative humidity atmospheres

Table I. Some Properties of Citrus Pellets Relating Weight, Area, and Volume (2)

Property	Pellet diam.	
	0.64 cm	0.95 cm
Pellets/kg	1500	1400
Area (cm ² /pellet)	4.5	4.6
Area (cm ² /kg)	7000	6500
Pellet vol (cm ³ /kg)	1010	1070
Bulk density (kg/m ³)	625.8	577.6
Void space (%)	63.3	60.4

dried citrus pulp, dark in color, is less palatable and has less nutritional value due to a reduced digestibility of protein and lower energy value. For comparison, the average nutrient composition of a large number of samples of commercial citrus pulp is presented in Table II.

When a study was made by Pulley and von Loesecke (8) of dried grapefruit pulp manufactured by three types of dryers, they found no significant nutritional differences between the products except where pressing was avoided. However, carotene was almost completely destroyed by the drying process. It has been demonstrated that the monohydroxy and dihydroxy-carotenoid fractions of orange and tangerine flavedo were easily destroyed during drying (9). Elevated drying temperatures increased pigment losses. Samples dried at exit-stack gas temperatures of 99 and 110°C were light in color and showed the least loss of pigment during drying. Extensive destruction of pigment occurred in other samples dried at an exit gas temperature of 143°C. Since 143°C is widely used in commercial feed mills, a reevaluation of citrus pulp drying temperatures is suggested.

Peel Oils

The economic importance of the various citrus oils has resulted in intensive research directed toward processing methods, compositional analyses, quality, and utilization. Early work described recovery methods and some characteristics related to quality of citrus peel oils in the United States (10, 11). More recently, citrus peel oil yield and quality has been related to processing methods (12) and cultural practices (13).

Chemical composition. Detailed compositional data concerning the commercially important citrus oils can be found in several reviews. An extensive list of various chemical compounds identified as components of orange, grapefruit, tangerine, lemon, and lime oils has been compiled and described in relation to certain flavor properties (14). Ziegler (15) described some gas liquid chromatography (GLC) techniques useful for analyses of essential oil components and reviewed considerable research relating specific compounds to important flavor characteristics of oils from sweet and bitter orange, bergamot, orange juice, mandarin, grapefruit, and limes. In a review of many essential oils and important flavor compounds, some discussion was devoted to citrus oil composition and certain analytical methods useful in comparing different oils (16).

Use of GLC to determine qualitative and quantitative composition of volatile compounds in citrus oils has become common. However, there are many discrepancies in the literature concerning the quantitative composition of citrus oils. Analyses of volatile aldehydes are of major importance, because composition and quantity of these compounds influence the quality and value of citrus oils. Compositional differences between

Table II. Average Nutrient Composition of Citrus Pulp Samples Collected During a 13-year Period (6)

Samples (N = 3630)	Nutrients (%)					
	Moisture	Ash	Ether Extract	Protein	Crude Fiber	N-free Extract
Air-dry Avg.	8.48	5.02	3.69	6.23	12.12	64.46
basis Range	2.0-18.4	3.1-11.1	1.1-11.6	4.9-9.3	6.4-17.8	54.2-72.3
Dry Avg.	0	5.42	4.08	6.80	13.32	70.38
basis Range	0	5.0-6.77	3.64-4.34	6.51-7.19	12.44-13.94	69.56-71.48

aldehydes in California, Florida, and Israeli oils have been shown (17). Coleman et al. (18) quantitatively estimated orange essence oil volatiles by GLC and showed a peculiar absence of octanal and decanal. Another report estimated the total amount of octanal plus hexanal at 1.29% in a volatile fraction of commercial Valencia essence oil, yet did not report the presence of decanal, a major aldehyde of commercial orange essences and oils (19). Other quantitative GLC data also shows considerable variation in the amounts of specific aldehydes, octanal and decanal, in Valencia orange oil (15, 17, 20, 21).

Certain values have been reported for the concentrations of oxygenated compounds in citrus oils using chemical methods, rather than GLC. Naves (22) reported 31% octanal, 27% decanal, 6% dodecanal, and 7.5% citral in sweet orange oil. More recently, the percentages of aldehydes, esters, alcohols, and acids present in terpenless citrus oils (Table III) have been determined (23).

Processing effects. Citrus oil quality may be affected by certain processing parameters. Some which have been studied include effects of yield from the fruit, the amount of water used during recovery and extraction, storage of fruit prior to processing, blending of fruit varieties, and handling the emulsion or finished oils (12).

The type or blend of fruit processed has a significant effect on oil quality. An important aspect of this variable which can be controlled would be to avoid processing mandarin or other varieties in with oranges. Subtle flavor differences, which can be detected by the flavorist, may be imparted to orange oil adulterated with tangelos, murcotts, temples, or tangerines. A mixture of mandarin fruits with oranges may be detected by ultraviolet spectra and results in inconsistent flavor quality and lower aldehyde contents.

The quantity of water used to make the oil-water emulsion also affects quality. Aldehyde concentrations decrease with increasing proportions of aqueous phase in an emulsion. This decrease may be due to insoluble solids which absorb aldehydes in the emulsion. However, using large quantities of water will increase oil yields. Therefore, a balance should be sought between aldehyde content, water usage, and oil yield. Current industry water usage for oil recovery varies widely above and below 28 l/100 kg fruit depending on the type of recovery equipment (24, 25).

Data by Waters et al. (26) have demonstrated that the type of commercial extraction had a significant influence on the tocopherol content and the evaporation residue of citrus oils. The tocopherol content of midseason orange oils followed the order: Brown peel shaver (216 ppm), FMC in-line extractor (126 ppm) and screw press (104 ppm). The method of extraction influenced both the evaporation residue and the tocopherol content of orange oil, the higher the evaporation residue, the higher the tocopherol content. Since tocopherol is a good antioxidant,

Table III. Concentrations of Classes of Oxygenated Compounds Present in Various Citrus Oils (23)

Citrus oil	% (wt/wt) of Total Oil			
	Aldehydes	Esters	Alcohols	Acids
Hamlin orange	1.48	0.35	0.55	0.13
Pineapple orange	1.20	0.26	0.40	0.11
Valencia orange	1.63	0.27	0.87	0.13
Temple orange	1.87	0.42	0.63	0.30
Dancy tangerine	1.10	0.25	0.47	0.24
Orlando tangelo	0.57	1.22	0.59	0.45
Duncan grapefruit	1.80	3.26	1.06	0.39
Valencia essence oil	1.38	0.91	0.84	0.04

the implication here is that processes may be important to production of stable oils. Those oils with more tocopherol would tend to be stable for longer storage periods.

Of cultural effects, fruit variety probably has the most influence on oil quality, particularly aldehyde content (12). The aldehyde content of coldpressed oil of oranges is highest when made from Valencia oranges. Mixtures of pineapple and seedling oranges yield oils with lower aldehyde content; while mixtures of Hamlin and Parson Brown varieties give oils with the lowest aldehyde content. The effect of fruit maturity has a major influence on the quality of citrus oils. For example, the aldehyde content of coldpressed Valencia orange oil decreases by 0.3 to 0.5% in a normal processing season, aldehyde content of the oil is lowest for immature and over mature oranges. Late bloom fruit will also give an oil of low aldehyde content. It is understandable that firm, mature fruit of good quality produces the best quality oil. Seasonal variation, rainfall, fertilization, irrigation, budwood, and rootstock also can affect quality and yield but will not be discussed here.

Distilled and folded oils. Of the distilled oils, some mention should be made of quality problems occurring during the production of d-limonene from peel press liquor or oil-water emulsions. These manufacturing processes are described in detail elsewhere (3). In particular, feed mill press liquor or emulsion handling can affect the quality and the yield of d-limonene. At one time in the industry, the press liquor was pasteurized during oil stripping prior to holding for feeding to molasses evaporators. Since the advent of waste heat recovery evaporators, press liquor handling needs considerable improvement in sanitation. For instance, the dilute press liquor is pumped at ambient temperatures to large storage tanks which feed the evaporator. These tanks may be cleaned once per season or they may never be cleaned. The result is fermentation of the raw press liquor which uses the most fermentable sugars, producing alcohol which is stripped off into the heat recovery evaporator condensate. Losses may be high if limonene is recovered from this condensate since it is not uncommon for the condensate to contain 10 to 20% alcohol. This alcohol may dissolve some of the limonene during the recovery process, resulting in problems when the aqueous condensate ends up at the waste treatment plant. Additionally, sour smelling aromas may be produced by microorganisms during handling of emulsions or press liquor. These components then may be present in the finished product.

In most instances, storage and handling of folded oils are the same as for most single-strength oils. The major stability difference is that the flavor of folded oils persists in products where high temperature is a factor, i.e., candy or baked products. The quality of folded oils is a function of the folding procedure. Oils folded by washing with 60% alcohol will differ from those folded by distillation under vacuum. For

distillation, the time, temperature, and vacuum have major effects on the quality and yield, as esterification and de-esterification reactions can occur. Losses also occur in the aldehyde flavor fraction as the level of folding increases. This is illustrated in Figure 3, wherein 40% loss of aldehydes has occurred at threefold concentration and over 50% loss at tenfold (27). This loss is quite time-dependent in a still under given temperature-vacuum conditions; thus, it is industrial practice to perform the distillation as rapidly as possible to minimize losses and quality changes.

Pulp-Wash Solids

Citrus fruit is processed into several product streams at the time of juice extraction. The extracted juice goes immediately to finishers where part of the ruptured juice vesicles, or pulp, is removed. Because there are still juice and soluble solids remaining in the finisher pulp, further processing allows recovery of this material (28, 29). The general process consists of mixing water continuously with the juice pulp from the finisher, allowing juice solubles to be leached, or distributed into the water, and finishing to separate the pulp and recovered solubles. The wash liquid so recovered may be centrifuged to remove particulates and then concentrated by evaporation. This process is known in the citrus industry as "pulp-washing" and the recovered solution as "pulp-wash". Pulp-wash concentrate is an integral part of the citrus by-products industry and is sold world-wide for use as cloud, flavor, or beverage base purposes.

Composition. Scientific data concerning liquids washed with water from orange juice finisher pulp was first published by Olsen et al. (30). They studied Brix/acid ratios, sucrose, reducing sugars, pH, pectic constituents, turbidity, pulp content, ascorbic acid, viscosity, and flavonoid content of experimental and commercial samples. Characterization of pulp-wash continued with publication of quality data (31), examination of pectic substances (32), microbiology (33), and comparison of pulp-wash with orange concentrate (34).

Generally, pulp-wash concentrates are similar in composition to orange juice concentrates. In fact, it is not uncommon for juice extraction overages to end up in pulp-wash during commercial operations. True pulp-washes in general do not have the good flavor or color of juice concentrates. Color scores are significantly low enough that, for the most part, pulp-wash color is poor. Color measurements have been used experimentally in attempts to measure the amount of pulp-wash present in true juice concentrates (35).

The composition of pulp-wash is such that, if both pulp-wash and juice are from the same batch of fruit, it may be included as a component of the product called concentrated orange

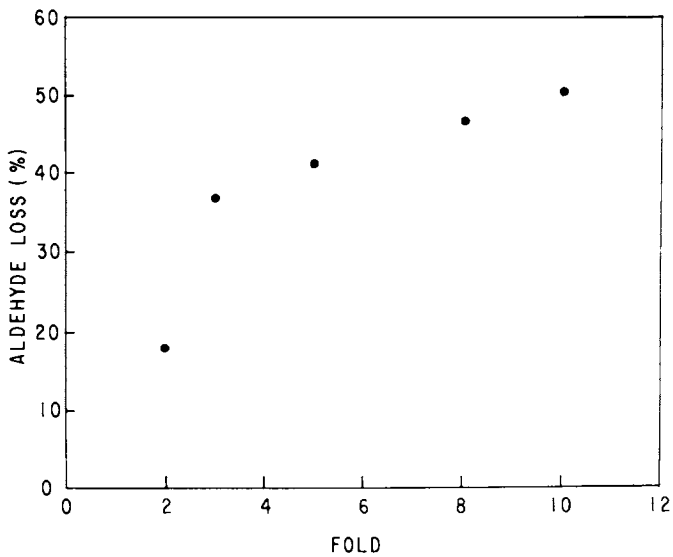


Figure 3. Loss of aldehydes during folding of cold-pressed oil (27)

juice for manufacturing (36). Under Federal law, the product, frozen concentrated orange juice, may contain water extracts of excess pulp (37); however, in Florida, the addition of pulp-wash solids to frozen concentrated orange juice is prohibited.

In a study of yield and recovery of various citrus by-products, it was reported that juice finisher pulp recovery was about 4.4 kg/100 kg of oranges and 2.7 kg/100 kg of grapefruit. Commercial recovery of soluble solids (ss) as pulp-wash should yield approximately 0.6 kg ss (oranges) and 0.2 kg ss (grapefruit) from 100 kg fruit (38).

Quality. Fruit and pulp conditions are major factors affecting pulp-wash quality. Handling difficulties are often experienced late in the season when very mature fruit are processed. If the pulp condition is soft or fragile, considerable water soluble pectin may be extracted during the counter-current washing process. This pectin causes liquids to be viscous, lowering extraction efficiencies in subsequent washing stages. Viscous pulp-wash liquids also present handling problems during concentration in the evaporators. To alleviate handling problems associated with high viscosities, processors now commonly add commercial pectolytic enzymes to pulp-wash liquids prior to concentration (39, 40, 41). These enzymes degrade the pectin, lower the viscosity of the liquids, allow concentration to a uniformly high ss content (50-65°B), and have very little effect on product quality (42).

Quality control of the process and of the final product is difficult, and varies widely from plant to plant. Generally, finisher pressures must be carefully controlled to avoid extracting undesirable flavors and pectic materials into the water phase. Process evaporation temperature should be considered an important parameter when good quality products are required. If liquids are too viscous, high temperatures may occur because of local overheating and poor heat transfer, resulting in a "cooked" flavor and browning caused by caramelization. If heat treatments are insufficient, natural or added pectinases in the liquids will remain active and products with poor clouding properties (and/or gelation problems) may be produced.

Some quality parameters, typical of commercial pulp-wash concentrates and dilute liquids, are included in the following statements:

For 60°B pulp-wash concentrate, a viscosity up to 10,000 cps (Brookfield with #4 spindle, 60 rpm, 26.7°C) and a #2 gel would be typical. Viscosities above 15,000 cps would be considered high, but the range 2,000 to 5,000 cps would be good if the liquids had been enzyme treated.

For a 10°Brix (°B) liquid reconstituted from 60°B concentrate, cloud measured initially on dilution should read not more than 20% light transmission (T) and more commonly in the range of 10% T. After 24 hr at room temperature, cloud values should

not be greater than 35% T. Sinking pulp in 10°B pulp-wash is usually less than 10% and most often is less than 5%. Clarification (separation of serum from cloud constituents) of 10°B liquids in a 100 ml graduate cylinder after standing 4 hr at room temperature will normally result in a separation of from 5 to 20 ml with enzyme treated liquids. At 10°B, serum viscosity is about 5 cps. A concentrate made from non-enzyme treated liquid and then diluted to 10°B might have a serum viscosity of 10 to 12 cps.

Recoverable oil (% by volume) of 10°B liquid reconstituted from concentrate is usually low (0.005 to 0.015%). Pulp-wash normally contains no floating pulp and should be reasonably free of hesperidin crystals and other defects. Pectinesterase activity should not exceed 5.0 P.E.U. (1 P.E.U. represents 1 meq. ester hydrolyzed per min per ml per °B) (43).

Pulp-wash concentrates are produced and handled in much the same manner as concentrated orange juices, and commonly are concentrated to 50 to 65°B. The primary use of the concentrates are in beverage base formulations as clouding agents and juice solids adjuncts. A major factor important to users of pulp-wash concentrates is the ease of handling during mixing or blending into the final beverage. Concentrates in the lower range of viscosities (2000 to 5000 cps) should meet this handling requirement. Flavors such as astringency or bitterness associated with immature fruit or peel juices should be avoided.

Dried Juice Sacs

The juice vesicles, or "sacs," remaining after juice extraction and pulp-washing may be included in the portion of peel residue dried as cattle feed. However, it is feasible to recover and utilize this material as either frozen (3) or drum-dried juice sacs (44).

Dried citrus juice sacs have excellent water and fat absorption capabilities, absorbing 10 to 12 times and 4 to 5 times their weight of water and fat, respectively. These absorptive properties make dried juice sacs valuable as emulsifiers or binders for comminuted meat products like luncheon meats, bologna, sausages, and frankfurters.

It has been proposed (44) that juice sacs can be processed into excellent thickening or bodying agents which could be used by the baking or food processing industries. Potential uses for juice sacs exist in the following specialty foods: canned or dehydrated gravies, sauces and puddings, preserves, cookie and pie fillings, pet foods, breading mixes for fried foods, and a variety of other food products. Dehydrated beverage mixes, synthesized juice products, and certain instant beverages also offer a potential use for dried juice sacs since frozen juice sacs are currently used in certain citrus containing liquid beverages. Drug formulations which utilize binders or water

absorbing materials are also envisioned as a possible market for dried juice sacs.

Drum dried-juice sacs prepared from either oranges or grapefruit have a very mild, bland flavor with an aroma that only faintly resembles the fruit from which they were prepared. The color of dried grapefruit juice sacs is white. Those prepared from oranges have a characteristic orange color which is unstable in the presence of light. Color, however, is not an important factor for most proposed uses of this product. If desired, the juice sacs could be dyed with certified food colors and used in many food products (45). When stored in opaque containers, the color will disappear in 3 to 4 months and become white making it difficult to distinguish between juice sacs from either oranges or grapefruit.

The chemical composition of dried orange juice sacs is as follows (44): crude fiber (18.9%), protein (9.0%), pectin (20.6%), ash (3.1%), fat 2.0%, moisture (10%), and other mostly carbohydrate material (36.4%). Another report (46), compared dried juice sac composition with whole peel and core material and found the three to be similar in composition.

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Methods for Determining the Quality of Citrus Juice

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In the thirty years from the middle of the 1930's to the middle of the 1960's the world experienced a growth in citrus processing and marketing that is hardly rivaled by any other food product. There was a slow steady increase in processed citrus from the middle 30's until shortly after World War II, when frozen concentrated orange juice was developed and successfully marketed. With the advent of frozen citrus concentrates the product acquired a new name--"The Cinderella Product," and it was. The manufacture of this product resulted in phenomenal growth; it did not occur easily or smoothly. Quality control methodology was being developed and constantly changed to keep up with advancing processing technology (1).

Today quality control technology is advanced, sophisticated and adequate to assure high quality under most conditions for all processed cultivars and their by-products (2).

In the middle 1970's the marketing of citrus concentrates started to change. In earlier years the producer shipped the retail product from his plant directly to the market. Now more and more bulk citrus concentrates are being shipped to widespread markets for remanufacture locally. Bulk concentrates are now manufactured and stored at a high degree of solids in large capacity tank farms awaiting shipment within the United States in tank trucks or internationally in 55 gallon drums. With this change in marketing, more emphasis is being put on developing methods to determine the composition and quality of the end product.

METHODS FOR DETERMINING QUALITY

Brix Determination

Introduction. Degrees Brix is a term used to designate the percent by weight of dissolved sugar in a solution. In citrus juices it is used to indicate the percent of soluble solids contained in the juice. This is one of the more important

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determinants of quality. The Brix of a juice is used as a factor in determining maturity of fruit, to establish various grades of quality, and is important in pricing of the commodity. Soluble solids may be determined either with a hydrometer or refractometrically. Hydrometer readings corrected for temperature will give a closer reading to the true soluble solids content of a juice than a refractometer. Because citric acid depresses refractometer readings, a correction for the amount of acid must be added. Refractometer readings must also be corrected for temperature (3). It has been shown (4) (5) (6) that many other corrections must be made to the refractometer to make the readings more comparable to the true soluble solids content of citrus juices. Corrected refractometer readings on single strength juices will usually read 0.1 to 0.3 degrees Brix higher than on corrected hydrometer readings.

Brix by Refractometer.

- A. Equipment.
 - (a) Refractometer with a Brix scale calibrated at 20°C.
- B. Procedure.
 - (a) Consult instruction manual supplied with instrument for procedures covering methods of calibration, reading and care.
 - (b) Clean and dry prisms using distilled water and soft tissue.
 - (c) Place sample on prisms with a wood or rubber applicator, making sure there are no included particles of pulp or air bubbles. Allow time for instrument and sample temperature equalization.
 - (d) Make reading, apply temperature and acid corrections from Table I and Table II.
- C. Example. If the refractometer reading is 44.8° Brix, the acid content of the sample is 2.5% and the refractometer temperature is 22°C the calculations are as follows:

$$\begin{array}{r}
 44.8^{\circ} \text{ reading from refractometer} \\
 .49 \text{ correction for 2.5\% acid} \\
 \underline{.16 \text{ temperature correction for } 22^{\circ}\text{C}} \\
 45.45^{\circ} \text{ Corrected Brix}
 \end{array}$$

Brix by Hydrometer.

- A. Equipment.
 - (a) Hydrometer calibrated 5 to 15° Brix at 20°C in tenths of degrees. The hydrometer should contain an internal thermometer with a temperature scale in degrees Brix.
 - (b) Hydrometer cylinder $\frac{1}{2}$ inch larger in diameter than the body of the hydrometer.

TABLE II
CORRECTION TABLE FOR TOTAL SOLUBLE SOLIDS DETERMINED
BY MEANS OF THE REFRACTOMETER IN SUCROSE SOLUTIONS
CONTAINING CITRIC ACID (2)

% Anhy Citric Acid	Add (%)	% Anhy Citric Acid	Add (%)	% Anhy Citric Acid	Add
0.5	0.11	13.0	2.55	25.5	4.77
1.0	0.21	13.5	2.65	26.0	4.86
1.5	0.31	14.0	2.73	26.5	4.93
2.0	0.40	14.5	2.83	27.0	5.02
2.5	0.50	15.0	2.92	27.5	5.11
3.0	0.60	15.5	3.01	28.0	5.19
3.5	0.68	16.0	3.10	28.5	5.26
4.0	0.78	16.5	3.19	29.0	5.36
4.5	0.88	17.0	3.28	29.5	5.45
5.0	0.98	17.5	3.37	30.0	5.52
5.5	1.08	18.0	3.47	30.5	5.60
6.0	1.17	18.5	3.55	31.0	5.69
6.5	1.27	19.0	3.64	31.5	5.77
7.0	1.37	19.5	3.73	32.0	5.85
7.5	1.47	20.0	3.82	32.5	5.94
8.0	1.57	20.5	3.91	33.0	6.02
8.5	1.67	21.0	4.00	33.5	6.10
9.0	1.77	21.5	4.08	34.0	6.19
9.5	1.87	22.0	4.17	34.5	6.27
10.0	1.97	22.5	4.26	35.0	6.35
10.5	2.07	23.0	4.34	35.5	6.44
11.0	2.17	23.5	4.43	36.0	6.52
11.5	2.27	24.0	4.52	36.5	6.60
12.0	2.37	24.5	4.59		
12.5	2.47	25.0	4.69		

B. Procedure.

- (a) Fill clean dry hydrometer cylinder to overflowing with deaerated juice (see text for deaeration method).
- (b) Lower hydrometer into juice giving the stem a slight spin. Allow time for hydrometer to stabilize and temperature equalization.
- (c) Read hydrometer across the liquid level to the bottom of the meniscus.
- (d) Remove hydrometer, read degrees Brix correction for temperature, and apply to original reading to obtain corrected Brix.

Deaeration

Introduction. Air becomes incorporated into juice during processing or upon reconstitution of concentrates. The incorporated air must be removed if the Brix of the juice is to be determined by hydrometer. It is essential to deaerate juice when determining the color with a colorimeter. The color is slightly less affected by air when visual comparisons are made.

Equipment. Assemble as in Figure 1.

- A. Vacuum source, such as a small electrical pump.
- B. 5000 ml wide mouth flask.
- C. Hoses, 3 hole rubber stopper, tubing, clamps.

Procedure.

- A. All clamps closed, draw a vacuum on large aspirator flask.
- B. Place intake hose into raw juice sample, open clamp and draw the juice up into the flask to remove air bubbles.
- C. Shut off vacuum source, break vacuum by leaving raw juice sample clamp open.
- D. Open second clamp and allow deaerated juice to flow into a container.

Acid Determination

Introduction. Citric acid in citrus juices may be determined according to the Methods of Analysis as given in the AOAC (7). However, analysts who run a large number of tests daily have altered the method to speed up the titration and make calculations easier. One of these alterations is to use 0.3125 N sodium hydroxide rather than 0.1 N alkali. The use of the higher normality alkali is desirable especially when titrating samples of high density concentrates. It is almost imperative when titrating lemon concentrates.

The acid content in concentrated citrus juices is determined as the total titratable acid calculated as anhydrous citric

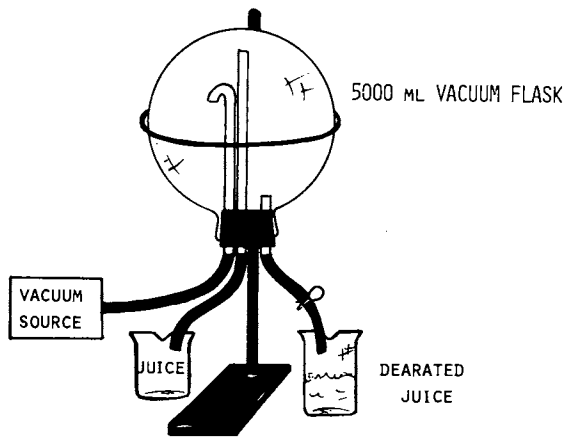


Figure 1. Deaeration apparatus

acid and is expressed as "percent by weight" (grams per 100 grams of concentrate).

Reagents.

- A. Sodium hydroxide solution 0.3125 N (12.5 g per liter sodium hydroxide reagent grade. Standardize against potassium acid phthalate).
- B. Phenolphthalein indicator solution - 1% solution in neutral isopropyl alcohol. This is prepared by dissolving 1.0 gram phenolphthalein powder in 100 ml of 50% isopropyl alcohol.

Equipment.

- A. Gram scale (accuracy \pm 0.05 g).
- B. 250 ml Erlenmeyer flask
- C. 50 ml burette
- D. Magnetic stirrer (optional)
- E. pH meter (optional)

Procedure.

- A. Weigh a 10 g sample of concentrate into a 250 ml Erlenmeyer flask.
- B. Add approximately 25 ml of distilled water and 1.0 ml phenolphthalein solution. If a magnetic stirrer is used, additional distilled water may be needed.
- C. Titrate with 0.3125 N sodium hydroxide solution to a definite pink color (end point), which holds for approximately 25 seconds or pH 8.2. As the end point approaches, the flavonones present in citrus juices will turn yellow.

Calculation.

- A. Multiply the number of milliliters of standard alkali used in the titration by 0.2. The result will be percent by weight of anhydrous citric acid by weight of concentrate. Example: 18.9 ml of standard alkali X 0.2 = 3.78% anhydrous citric acid.

The acid content in single strength citrus juices is determined the same way as in the procedure given above for concentrates using a weighed sample. However, a larger sample should be used. Using a 20-gram sample of single strength the number of milliliters of standard alkali should be multiplied by 0.1 to give the percent citric acid. Example: 9.7 ml of standard alkali X 0.1 = .97%.

Many analysts prefer to measure a given volume of single strength juice, instead of weighing it, then calculate the results on a weight to weight percentage. Example: Using a large opening 25 ml pipette, transfer a sample into a 250 ml Erlenmeyer flask and titrate to an end point the same as for concentrates. To calculate grams citric acid per 100 ml of juice, multiply the

milliliters of alkali by .08 to get grams citric acid per 100 ml of juice - weight/volume. The percent by weight can now be found using the following calculation:

$$\frac{\text{acid wt/vol.}}{\text{Specific gravity of juice}} = \text{Percent acid (wt./wt.)}$$

$$\text{Example: } \frac{\text{acid } 0.98 \text{ g/100 ml}}{\text{Sp.gr. } 1.050} = 0.93\%$$

The United States Standards for citrus juices, except lemon or lime products, specify that the citric acid determination (factor for citric acid equals 0.064) be calculated as grams per 100 grams juice. e.g.

Single-Strength Juices:

$$\frac{\text{Titer X } 0.3125 \text{ N X } 0.064 \text{ X } 100}{25 \text{ ml sample X specific gravity}} = \text{g per } 100 \text{ g}$$

Concentrates - except lemon:

$$\frac{\text{Titer X } 0.3125 \text{ N X } 0.064 \text{ X } 100}{10 \text{ g sample}} = \text{g per } 100 \text{ g}$$

The acid in frozen concentrate for lemonade and concentrate for limeade is specified as grams per 100 ml of the prepared lemonade or limeade. e.g.

$$\frac{\text{Titer X } 0.3125 \text{ N X } 0.064 \text{ X } 100}{25 \text{ ml lemonade or limeade}} = \text{g per } 100 \text{ ml}$$

The acid in concentrated lemon juice for manufacture is expressed as grams per liter. The easiest way to determine the acid in this product is to determine the acid in grams per 100 grams and convert this to grams per liter. e.g.

$$\frac{\text{Titer X } 0.3125 \text{ N X } 0.064 \text{ X } 100}{5 \text{ g concentrate}} = \text{g per } 100 \text{ g}$$

$$\text{Acid g/100 g X specific gravity X } 10 = \text{g per liter}$$

The Food and Drug Standards of Identity specify that citric acid be determined as grams per 100 ml of juice. e.g.

$$\frac{\text{Titer X } 0.3125 \text{ N X } 0.064 \text{ X } 100}{25} = \text{g per } 100 \text{ ml}$$

There are burettes on the market which are direct reading and are marked "percent Anhydrous Citric Acid." These burettes are used by the Florida State Department of Agriculture in their fresh fruit inspection work. This calibration of the burette is

made assuming that the specific gravity of the juice is always 1.0400 or 10.0 degrees Brix. The direct reading burettes are calibrated using the following formula:

$$\frac{\text{Titer} \times 0.3125 \text{ N} \times 0.064 \times 100}{25 \times 1.04} = \text{percent acid}$$

Brix-Acid Ratio

The Brix-acid ratio is a very important calculation used in the citrus industry. It is used as an index of maturity of fruit, it is an important factor in establishing the difference between grades in United States Standards, and it is an indication of the relative tartness or sweetness of juice. A ratio is calculated by dividing the Brix of the juice by the percent citric acid that it contains. For example: A sample of single strength 12.5° Brix \div 0.85% acid = 14.7 ratio, or of concentrate 44.85 Brix \div 3.05% acid = 14.7 ratio. 14.7 ratio means that for every 14.7 parts of soluble solids there is one part acid. The lower the ratio the more tart the juice tastes, the higher the sweeter. The ratios of mature fruit vary widely among the kinds of fruit and various climates throughout the world. Grapefruit juice having a 9.0 or 10.0 ratio would be considered sweet whereas this would be very tart in oranges.

Recoverable Oil (Scott Oil Method)

Introduction. Citrus essential oil is one of the most important constituents that contribute to the flavor of juice. Too little oil and the product tastes flat and lacks character; too much and the product gives the mouth a burning terpen-like sensation. United States Standards for Grades of citrus juices regard too much oil as a defect in manufacturing. The official method for the determination of recoverable oil is the Scott Method (8).

Reagents.

- A. Standard bromide-bromate solution, prepared and standardized to 0.099 N in accordance with Standard Solutions given in the AOAC (7). For use, add 1 volume of standard solution to 3 volumes of water to make 0.0247 N solution. 1 ml of 0.0247 solution supplies bromine to react with 0.00085 g or 0.0010 ml of d-limonene. The solutions are stable for six months.
- B. Isopropyl Alcohol - reagent grade.
- C. Diluted hydrochloric acid (2H₂O: 1HCl) or alternately 600 ml concentrated HCl with 1200 ml H₂O adding 9 ml 0.1% methyl orange indicator.
- D. Methyl orange indicator 0.1% in water.

Equipment. (assemble as in Figure 2)

- A. 500 ml boiling flask, flat bottom with a 24/40 joint connecting tube.
- B. 200 mm Graham condenser with 28/15 receiving socket and drip tip.
- C. 25 ml fast delivery pipette.
- D. Connecting tube adapter 24/40 drip tip, 28/15 ball, with inclined glass plate.
- E. 150 ml beaker Griffin low form.
- F. Magnetic stirrer and bar.
- G. Electric heater with recessed refractory top, 750 watts.
- H. 25 ml burette graduated to 0.1 ml.
- I. Ball joint clamp.

Procedure.

- A. Pipette 25 ml sample of juice or reconstituted juice into the distillation flask containing glass beads.
- B. Add 25 ml of isopropyl alcohol.
- C. Distill into a 150 ml beaker. Continue distilling until solvent ceases to reflux.
- D. Add 10 ml of diluted HCl solution with indicator to the distillate.
- E. Titrate while stirring with 0.0247 N bromide-bromate solution. Disappearance of color indicates the end point.
- F. Determine the reagent blank by titrating three separate mixtures of 25 ml isopropyl and 10 ml of dilute hydrochloric acid with indicator - without refilling the burette. Divide the total milliliters of titrant used by three to obtain the average blank. Subtract the average blank obtained from the milliliters of titrant used to titrate the distillate.

Calculations. One milliliter of titrant is equal to 0.004% oil in the 25 ml sample. For example, if 2.9 ml of the bromide-bromate solution is used and the average blank of the reagents is 0.1 ml the percentage of oil in the sample would be $3.2 \text{ ml titrant} - 0.1 \text{ ml blank} = 3.1 \text{ ml}$ $3.1 \text{ ml} \times 0.004 = 0.0124\%$. The oil content is expressed as percent by volume.

Pulp - Free and Suspended

Introduction. This test is commonly called centrifuge pulp or fine pulp. The amount of centrifuge pulp contained in frozen concentrated orange juice is regulated in Florida. It is a factor in the U. S. Standards for Grade of grapefruit juice. Large amounts of centrifuge pulp in a sample is an indication of high extractor and finisher pressures applied to the fruit during manufacture. It may also indicate the softness or freeze damaged fruit.

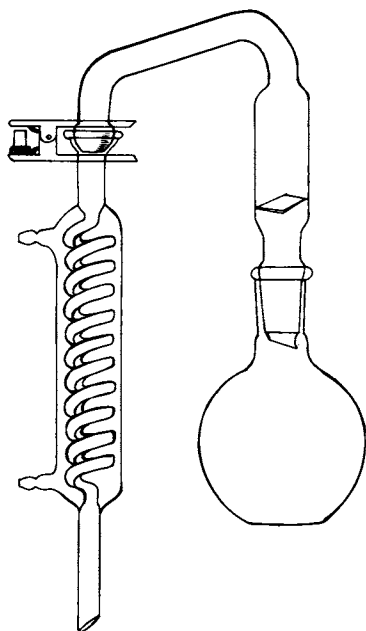


Figure 2. Oil distillation apparatus

Equipment.

- A. Centrifuge
- B. Accurate tachometer - a vibration type tachometer measuring 1500 rpm graduated in increments of 25 rpm or less is recommended.
- C. Variable voltage transformer (optional).
- D. Electric timer with automatic cut-off switch.
- E. 50 ml graduated centrifuge tubes with short conical bottoms.
- F. 20 mesh stainless screen or a nominal 20 mesh stainless steel tea strainer.

Procedure.

- A. Reconstitute all orange juice concentrates to 11.8° Brix and all grapefruit juice concentrates to 10.0° Brix. Single strength juices only need to be well mixed.
- B. Bring the temperature of the product to 80°F ± 2°F.
- C. Remove coarse and floating pulp by pouring through a 20 mesh screen.
- D. Fill two centrifuge tubes to the 50 ml graduation with juice, and place the tubes in the centrifuge so the graduated scales face the direction of rotation.
- E. Adjust the speed up to the required revolutions per minute in accordance with centrifuge speed chart given in Table III, and centrifuge the juice for 10 minutes (9). Note: Do not put centrifuge on full speed and adjust down to the required rpm.

Calculations. After centrifuging the pulp surface will probably be uneven. Average the milliliter reading at the top of the layer of pulp at its highest point and also at its lowest point in each tube to obtain the amount of suspended pulp in each tube. The averaged reading multiplied by 2 will give the percentage of suspended pulp by volume.

Pulp-Floating

Introduction. Whole and large pieces of juice sacs from the fruit are termed by the trade floating pulp. This type pulp is generally removed in the early stages of juice processing and added back at a later stage. The amount of floating pulp can vary widely among manufacturers because different distributors have a definite preference for the amount in their juice (2). Floating pulp is added back to juice products to promote mouth feel and eye appeal. The current regulations and U. S. Standards consider floating pulp only if the pulp is present in large amounts to adversely affect the drinking quality of the juice.

TABLE III
CENTRIFUGE SPEED CHART (9)

<u>Diameter</u> <u>(inches)</u>	<u>Approximate</u> <u>revolutions</u> <u>per minute</u>
10	1,609
10½	1,570
11	1,534
11½	1,500
12	1,468
12½	1,438
13	1,410
13½	1,384
14	1,359
14½	1,336
15	1,313
15½	1,292
16	1,271
16½	1,252
17	1,234
17½	1,216
18	1,199
18½	1,182
19	1,167
19½	1,152
20	1,137

Diameter means the overall distance between the bottoms of opposing centrifuge tubes in operating position.

Equipment.

- A. A nominal 20 mesh stainless steel tea strainer.
- B. Gram scale.

Procedure.

- A. Concentrates, reconstitute to 24 fluid ounces; other juices, use 32 fluid ounces.
- B. Pour the entire volume through the strainer and shake by hand until the pulp retained on the screen "balls up" and is free of excess juice. Dry the bottom of the screen to remove adhering juice.
- C. Turn the ball of pulp onto a clean dry scale pan, disregarding any slight amount that clings to the strainer.

Calculations. Record the weight of the pulp in grams.

Naringin Analysis (Davis Test)

Introduction. Naringin is a bitter flavanone glycoside found in grapefruit. It is not the only bitter compound in grapefruit, but it is used by the industry as an index to the degree of bitterness for grapefruit juice. The Davis Test (10) is a fast test for total flavanones in grapefruit juice. It is not specific for naringin; however, it is a valuable index of the amount of naringin. Kesterson (11) determined that 90 percent of the naringin is contained in the albedo and membrane of grapefruit. Since this is the case, higher naringin contents in juice is caused by increased extractor and finisher pressures. Maurer (12) found that samples of juice which contained more than 70 mg possessed an immature bitter taste. Grapefruit juice containing less than 50 mg seemed to have a superior flavor. Until recently it was customary to refer to naringin content as milligrams percent. Now concentrations are being expressed as parts per million.

Reagents.

- A. Diethylene glycol (2,2 Oxydiethanol) 90% reagent grade. Commercial grade may be used if it is clear without a tinge of yellow.
- B. Purified naringin (Eastman)
- C. Sodium hydroxide 4N

Equipment.

- A. Colorimeter or Spectrophotometer with 420 nm wavelength.
- B. 1 ml serological pipettes, graduated in tenths of a ml.
- C. Centrifuge and centrifuge tubes.
- D. Test tubes with neoprene lined screw caps 25 mm X 150 mm.

Procedure for Standard Curve.

- A. Weigh 250 milligrams naringin and suspend in 175 ml of distilled water. Warm solution on water bath until dissolved. Let cool and make up to 250 ml in a volumetric flask. This is a stock solution.
- B. From the stock solution prepare solutions containing 10, 20, 30, 50, and 75 mg of naringin per 100 ml of distilled water.
- C. In each of five test tubes, place 25 ml of 90% diethylene glycol. Take 0.5 ml of the 10 mg standard solution add to one of the tubes. To the second add 0.5 ml of the 20 mg standard solution and so on until a series of 5 tubes containing increasing amounts of naringin are prepared. Add 0.5 ml of 4N sodium hydroxide to each tube. Mix well by tilting back and forth. Do not shake. Let the tubes stand for 10 minutes for the yellow color to develop.
- D. After 10 minutes transfer the samples to cuvettes and read the absorbency at 420 nm. Use diethylene glycol as a blank. Plot absorbency vs milligrams of naringin per 100 ml. This should yield a straight line on arithmetical graph paper.

Procedure for Determination.

- A. If the sample is a concentrate, reconstitute to 10.0 Brix. Mix samples well and bring to room temperature.
- B. Place sample in a centrifuge tube and centrifuge for 10 minutes. Centrifuge speed is determined from Table III.
- C. Place 25 ml of 90% diethylene glycol in a test tube, add 0.5 ml of supernatant juice, and 0.5 ml of 4N sodium hydroxide. Mix well by tilting the tube back and forth. Do not shake. Let stand 10 minutes for color development.
- D. Read absorbency at 420 nm. Read naringin from curve.

Defects

Introduction. Defects in citrus juices are manufacturing errors that affect the appearance or palatability of the product. Defects usually take the form of inclusions of harmless material that, in a small degree, may be unavoidable. The definition of a defect does not include any type of foreign material, the inclusion of which is totally unacceptable.

Citrus juices are produced to have, as nearly as possible, the character and appearance of freshly extracted juice with a normal amount of juice cells and a slight amount of pulp. Occasionally very small seeds, or portions thereof, are expected. Only very small seeds or small fragments that could pass through round perforations of no more than 1/8 inch in diameter are

acceptable and then only in a small degree. Seeds or seed particles larger than this are indicative of poor manufacturing practices.

The glycoside hesperidin is a naturally occurring component of juice from oranges, tangerines and many of their hybrids. Under certain conditions of pH and fruit variety it precipitates as a scale or coating in the evaporators. When this scale becomes thick or heavy, small portions peel off and become noticeable as small white or yellow flakes in the finished product. This compound will not go back into solution and since these particles tend to settle rapidly, their presence in the reconstituted juice can be quickly detected.

Occasionally citrus juices will contain a few dark specks. This is generally caused by juice or juice sacs becoming scorched or burned either during pasteurization or evaporation. Dark specks are objectionable and any given sample of juice should contain very few. All dark specks should be examined with a wide field microscope to determine that the specks are scorched pulp and not some other kind of material.

Excessive citrus oil is considered a defect. During processing the amount of oil in citrus juices can be controlled; therefore, any excess amount is considered a manufacturing error.

Citrus juices have a very high potassium content, and in recent years monopotassium citrate, on occasion, has recrystallized in high density concentrates. This recrystallization occurs after production and during storage. The occurrence of the crystals is sporadic and unpredictable. Concentrates containing crystals have a very grainy appearance. It is undesirable to manufacture a retail concentrate product from concentrates containing these crystals because they will not go back into solution. It is felt that consumers would think that these crystals are foreign material of some kind. High density concentrates containing potassium citrate crystals are acceptable for manufacturing a reconstituted product since the crystals will then be redissolved.

All U. S. Standards for grades of citrus products take into account, with a score point system, the degree of freedom that the product has from defects. The higher grade of products must be "practically free of defects," while the lower grades must be "reasonably free from defects" (19).

Procedure.

- A. Reconstitute an amount of concentrate at the correct degrees Brix (11.8° for orange and 10.0° for grapefruit) that will result in 24 fluid ounces of product, or the same sample size of well mixed single strength juice.
- B. Pour into a standard 1000 ml glass beaker (bottom diameter 4 inches) and let stand five minutes.

- C. Examine juice for defects by holding the bottom of the beaker above a strong light.

Color Determination

Introduction. A pleasing bright normal color in food products is essential to the enjoyment of eating. That color is a psychological, as well as a physical, fact has been well established. It is also known that normal color vision is three-dimensional (13). A spectrophotometric curve may be reduced to three numbers, which will adequately and accurately describe the color of any substance. There are a number of systems using the three-dimensional concept to define colors as tristimulus values. The standard system, against which others can be compared, is the one recommended by the International Committee on Illumination and is based on the "standard observer." This can be thought of as a simulated standard eye, consisting of three primary color filters (14). These filters have exactly specified spectrophotometric curves, X, Y, and Z; with X being amber in color, Y green, and Z blue. Hunter (15) developed a tristimulus colorimeter that gained wide acceptance in the food industry. He also developed the Hunter L, a, b scale. This scale is easier to identify with visual color than the X, Y, Z scale. The L values are directly comparable to the value Y, the a values are measures of redness or greenness, b values are measures of yellowness or blueness (see Figure 3). The a values are functions of X and Y, and b values are functions of Z and Y.

$$L = 100 \sqrt{Y}, \quad a = 175 \frac{(1.20 X - Y)}{\sqrt{Y}}$$

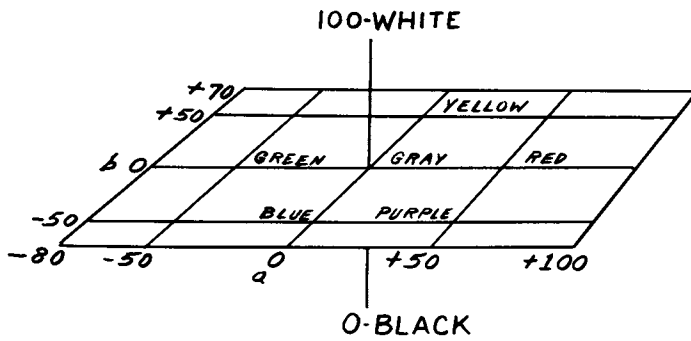
$$b = 70 (Y - 0.847 Z/Y)$$

Many single number color scales have been developed for foods by converting X, Y, Z or L, a, b color specifications to a number indicating grade or quality on the desired scale. Such a scale was developed by Hunter to measure the citrus redness CR, and the citrus yellowness CY of the juice. The formulae are derived as follows:

$$CR = 200 \frac{(1.277 - 0.213Z - 1)}{Y}$$

$$CY = 100 (1 - 0.847 Z/Y)$$

Huggart (17) using a Hunter Citrus Colorimeter developed an equation containing CR and CY values which established a high correlation between instrument color values and U. S. Department of Agriculture visual color scores. The State of Florida



HunterLab

Figure 3. Rectangular surface-color solid with dimensions L , a , b

adopted this method and promulgated regulations making the colorimetric method mandatory for grading orange juice products (18). The measurement of color on tristimulus colorimeters will depend on the type of machine and its operating instructions. It should be noted that orange juice color readings are affected by incorporated air and by variations in temperature. The juice should be deaerated and brought to $80^{\circ}\text{F} \pm 2^{\circ}$ before readings are made. Color value readings will have to be correlated with visual evaluations to be meaningful.

Orange juice ranges in color from a very pale yellow to a very rich orange depending on variety. The very orange color is considered the most desirable. The Federal Standard of Identity allows the mixing of juice from tangerines and its hybrids with the juice from sweet oranges in amounts not to exceed 10 percent by volume. Since tangerines and its hybrids are so highly colored, producers mix the allowed volume to improve the color of the lighter juices.

The U. S. Grade Standards for orange juice (19) products describes different levels of quality depending upon the color classification. According to USDA standards "Very Good Color" means a very good yellow to yellow-orange color that is bright and typical of rich-colored fresh orange juice. "Good Color" means that the color is yellow to yellow-orange, typical of fresh orange juice which may be dull but not off color. "Reasonably Good Color" means the color is yellow to yellow-orange which is reasonably free from browning due to scorching, oxidation, carmalization or other causes. Blood orange juice should be a pure red without any traces of dullness or browning.

Evaluation of Color.

- A. The color of orange juice is evaluated by comparing the color of the single strength or the properly reconstituted product with the USDA orange juice color standards, so that these color standards become points of reference.
- B. The comparison is made under an artificial light source of approximately 150 candela intensity and a color temperature of 7500 degrees Kelvin \pm 200 degrees.
- C. The USDA orange juice color standard consists of six plastic tubes which range from a strong orange color to a light yellow. USDA OJ 1 tube is the most orange color in the series with OJ 6 tube being the lightest.

Procedure for Evaluating Color.

- A. Place the juice sample into a glass test tube one inch in diameter.
- B. Arrange color standards and sample in a test tube rack or similar device so that light coming from above strikes the standards at a 45 degree angle. The standards are inclined against a neutral grey background. Observe the standards and samples at right angles to the tubes.

- C. Classify the juice by inserting the sample tube where it fits best in the series of color standards. See Table IV.

Availability of Color Standards. The USDA orange juice color standards cited in this section are official color standards. Information regarding the color standards and their availability may be obtained from:

Chief, Processed Products Branch
Fruit and Vegetable Quality Division, FSQS
U. S. Department of Agriculture
Washington, D. C. 20250

Grapefruit Juice Color. Grapefruit juice ranges in color from pale yellow to a very slight amber for white-fleshed grapefruit, or slightly pink to a distinct red for red-fleshed grapefruit juice. Immature white-fleshed grapefruit yields a juice which is oyster white with possibly a very slight greenish cast. As pink or red-fleshed grapefruit ripen, the color of the juice loses the distinct coloration and gives a juice that is definitely amber with a very slight brownish cast. Mixtures of white-fleshed and pink-fleshed grapefruit have a dull appearance and may look grayish to dull amber. There are no visual color standards for grapefruit juice products. However, color characteristics may be measured on tristimulus colorimeters using the L, a, b, color notation.

Enumerating Microorganisms

Introduction. The importance of microorganisms in certain citrus products should not be underestimated. Especially vulnerable are citrus concentrates, pasteurized citrus juices, citrus juice from concentrate and chilled citrus sections. The composition of citrus juices presents a highly selective medium for growth of microorganisms. It has a low pH, a relatively high ascorbic acid content, and contains citrus peel oil. Because of the acidity and low pH values the growth of most types of bacteria is prevented and pathogens (disease-producing microorganisms) are of no consequence (2). However, there are a number of kinds of microorganisms that survive processing and appear in the finished product (20). Product that contains a high bacteriological count is often thought of as being produced under unsanitary conditions. In spite of the usual precautions, such as plant sanitation and efficient washing and grading of fruit, the use of over-mature, low-acid fruit will often result in a high-count product (21). When concentrates are first produced, bacteria rather than yeasts are the predominating organisms present. The bacteria die fairly rapidly in storage

TABLE IV
SCORING GUIDE FOR COLOR
IN VARIOUS PROCESSED ORANGE JUICES

	USDA OJ 1 Equal to or better than	Not as good as OJ 1; better than OJ 2	USDA OJ 2 Equal To	Not as good as OJ 2; better than OJ 3	USDA OJ 3 Equal To	Not as good as OJ 3; better than OJ 4	USDA OJ 4 Equal to or slightly better	Not as good as OJ 4; better than OJ 5	USDA OJ 5 Equal To	Not as good as OJ 5; better than OJ 6	USDA OJ 6 Equal To	Not as good as OJ 6
ORANGE JUICE												
Frozen Concentrated												
Pasteurized												
From Concentrate	40	40	40	39	39	38	37	36	36**	35	34	33 or less
Canned Concentrate												
Canned	40	40	40	39	39	38	38	37	37	36**	36**	35 or less
Concentrated for Manufacturing												
Dehydrated	40	40	40	39	39	38	37	36	36	35	34**	33 or less

**Limits for U. S. Grade A

CAUTION - Concentrate must be reconstituted correctly
before color evaluation.

but yeast count will be relatively the same even after eight months storage at below 0°F (22). Even though there are various types of organisms that survive the manufacture of citrus juices, most of them will not grow or reproduce. The microorganisms in the juice that are capable of growing, even at refrigerator temperatures, are Leuconostoc, Lactobacillus and various genera of yeasts (23). These organisms, given the opportunity, will develop off-flavors and off-odors in juice. The Leuconostoc and Lactobacillus produce diacetyl which gives the juice a strong "buttermilk" like flavor and odor. Yeasts will cause a sour, alcoholic flavor and odor with gas production. Several species of the Lactobacilli will grow at relatively high temperatures. One strain has been isolated from the regenerative section of a heat exchanger which grew very rapidly in single strength juice at 49°C (24). The enumeration of bacteria before and after heat exchangers is especially important when reprocessing bulk concentrate into citrus juice from concentrate.

Standard Plate Count. This is a means of estimating the number of viable bacterial cells per given quantity of juice and is generally reported in terms of the number of microorganisms per ml of concentrated juice, single strength juice or the packing medium of chilled sections.

It is of the utmost importance to insure that aseptic technique is used throughout these procedures. The work place must be suitable for bacteriological work. It is very difficult to produce meaningful work where there are air currents and laboratory traffic. Normal air contains bacteria and mold spores which can easily contaminate samples and plates.

Medium Preparation and Sterilization. Orange Serum Agar has been developed specifically for the bacteriological examination of citrus products. It gives a high total count of microorganisms and is especially recommended for the enumeration and cultivation of organisms causing spoilage in citrus products. Dehydrated medium is available commercially and should be prepared according to the directions given by the manufacturer.

The prepared medium is dispensed into screw cap bottles (prescription ovals 125 ml to 250 ml capacity), which should never be filled more than two-thirds full. Bottles of the freshly prepared medium are capped loosely and sterilized in an autoclave or pressure cooker for 15 minutes at 15 pounds pressure (120°C). When the medium has been sterilized and cooled the caps should be tightened upon removal from the autoclave or pressure cooker, and stored in a cool place.

Sampling Containers. Pre-sterilized disposable sampling containers are preferred for the collection of samples. Screw capped bottles may be used if they are first sterilized under 15 pounds pressure for 15 minutes. If glass bottles are

sterilized they should contain about 1 ml of water per bottle. Bottles which are dry inside cannot be considered sterile at this recommended time and pressure. Samples should be taken directly in the sterilized container. Sterile metal sampling dippers may be used to remove samples from vats or tanks. Samples should not be stored but should be plated immediately after sampling, as storage might increase the counts. Samples which cannot be plated immediately must be chilled rapidly and thoroughly.

Pipettes and Petri Dishes. It is preferable to use pre-sterilized disposable pipettes and petri dishes. They are not only more economical to use, but the method of sterilizing and sealing is generally better than that accomplished in most laboratories. Pipettes should be 1 ml serological type graduated in 0.1 ml. If concentrates are to be sampled, the large bore pipette for viscous liquids should be specified. Petri dishes should be standard 100 x 15 mm.

Sterilization of Dilution Bottles. Pyrex milk dilution bottles with screw caps are preferred. The bottles are filled with 99 ml of water and sterilized under 15 pounds pressure (121°C) for twenty minutes. Bottles should not be opened except before and after delivery of the sample.

Dilution of Samples. Samples are generally plated in dilutions of 1-100 or 1-1000. The dilution used will depend upon the number of colonies present on the plates after a 48-hour incubation period, or what previous experience with the product has shown. Dilutions should be adjusted so that the plate counts fall between 30 and 300 colonies per plate. If the count falls outside this range, higher or lower dilutions are necessary. Upon initial plating, when the dilution range is unknown, samples should be plated in dilutions of 1-100, 1-1000, 1-10,000 and made in duplicate. If spoilage is suspected the lower dilutions are not necessary and even higher dilutions made.

To prepare dilutions, 1 ml of concentrate, single strength juice, or packing medium from chilled sections is removed from the sample and transferred to a sterile 99 ml water blank. This gives a 1-100 dilution. The 1-100 dilution is shaken vigorously at least 25 times over a 7 second period. This is to break any bacterial clumps that might be present. Using a sterile pipette 0.1 is transferred to a petri dish for the 1-1000 plate. 1 ml is transferred to another petri dish for the 1-100 plate. To prepare a 1-10,000 dilution, 1 ml of the 1-100 dilution is transferred to a sterile 99 ml water blank and shaken as described. 1 ml of this dilution is delivered to a petri dish for the 1-10,000 dilution and 0.1 ml would be a 1-100,000 if this high a dilution is needed. When plating packing media from chilled sections

that contain preservatives lower dilutions may be needed. 1 ml of the packing medium transferred to a petri dish will give a 1-1 dilution.

Bacteriological Procedures. Microorganisms multiply rapidly under ideal conditions. Consequently, it is necessary that samples be plated as soon as possible after they are drawn. If the samples are in a frozen state, they should be thawed in tepid water. If there are a large number of samples, they should be thawed in workable groups. It is not advisable to allow a sample to stand over an hour. If the samples are in twist top sterile bags or screw cap bottles they should never be immersed in water. If the sample is in a can it should be dried thoroughly, the top swabbed with 70 percent alcohol and opened with a beer opener that is still warm from flaming.

It is essential that from the puncturing of the can or opening the sample through to the plating, extreme caution is to be exercised in aseptic technique. The mouth of each dilution bottle must be flamed prior to and after delivery of the sample. Caps on the bottles must be carefully removed and held between the fingers in such a position that the bottom or inside of the cap does not touch the fingers. If this happens in any way the bottle must not be used. This applies to pipettes as well. Pipettes can only be used once.

Prior to plating a sufficient amount of agar must be melted. Place the required number of bottles in a sauce pan and fill with water two-thirds the height of the bottles, boil the water until the agar is completely melted. After melting the agar is cooled and maintained at a temperature of 45°C until used. It is very important that the agar is not hot when poured since microorganisms are very susceptible to high temperature. The procedure is as follows:

- A. Using a sterile pipette aseptically transfer the sample to a dilution bottle, the mouth of which has been flamed. Flame the mouth again and replace cap.
- B. Shake the dilution bottle at least 25 times.
- C. Remove the dilution bottle cap, flame and remove a sample with sterile pipette. Transfer this to a flamed dilution bottle or a sterile petri dish. The petri dish cover should not be fully removed, just tilted up enough that the pipette can be inserted.
- D. The cap is removed from the agar bottle, the bottle flamed and approximately 15 ml is poured into the petri dish with the sample. The sample should be mixed carefully with the agar by a slow rotation of the plate, in a figure 8 pattern. The agar should not be allowed to slosh up on the rim or top of the dish.
- E. The agar plates are allowed to harden prior to incubation.

- F. When the agar is hardened the plates are inverted and placed in an incubator for 48 hours at 30°C.
- G. A control plate should be run with each batch. This consists of pouring agar into a petri dish containing 1 ml of the sterile dilution water. This gives a check on the sterility of glassware, medium, and dilution water.

Counting the Plates. After the plates are incubated for 48 hours the counts may be made. A Qubec Colony Counter facilitates counting the plates. It supplies the right lighting and magnification to detect and count pinpoint colonies which otherwise might be overlooked. The total plate count is arrived at by counting the number of colonies and multiplying that number by the dilution factor. For example, if there are 30 colonies on the plate, the dilution of which is 1-100, the total count for that plate would be 3000 microorganisms per milliliter. If duplicate plates are made (it is advisable), both are counted and the average count is reported.

It is not very likely that plates made in duplicate will show the same counts. Some plates may vary as much as 10 colonies per dilution. However, if there is a wider variation in the total counts on duplicate plates, it is more likely that the technique is at fault and should be thoroughly checked from beginning to end. Plates containing less than 30 colonies or more than 300 do not give a valid count. When this is encountered other dilutions should be made.

Significance of Counts. The total plate counts will vary throughout the season depending on the condition of fruit. Generally speaking, the higher the ratio of the fruit the more likely the higher the count. Normal counts on 45° Brix orange concentrate will vary from 25,000 to 75,000 microorganisms. A count of 100,000 is considered excessive.

Chilled or pasteurized juices packed in cartons with a total plate count of 300 to 500 organisms per milliliter, if handled properly, can expect a shelf life to three weeks. Chilled juices with a count of 5,000 per milliliter probably will not have a shelf life of more than a week.

Use of the permitted preservatives sorbates and benzoates will inhibit the growth of microorganisms but will not appreciably extend the shelf life of chilled citrus juices or chilled sections that have a heavy bacterial load.

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Problems in Sensory Evaluation of Citrus Products

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As in most food industries, the citrus industry in Florida is interested at all times in a thorough knowledge of the flavor quality of its products. To obtain this information, use of some sort of sensory evaluation is often employed. This may be by an experienced quality control person or group, a government food inspector, a small experienced group of judges gleaned from the technical plant personnel, a larger consumer-type sensory panel made up of diverse plant personnel, or perhaps a combination of the above. Most plants are also vitally interested in flavor quality of product manufactured elsewhere, including states other than Florida and foreign countries, including Brazil, already a citrus giant.

Of course, to control quality, raw product coming into the plant requires at least a cursory flavor evaluation by plant personnel in addition to objective tests which normally are run such as degrees Brix, % acid, color, etc. In Florida, all fruit delivered to a fresh fruit packer or processor is inspected by personnel from the Florida Department of Agriculture, Division of Fruit and Vegetable Inspection. Juice content and degrees Brix to % acid ratio are used in determining maturity according to specific standards during fresh fruit inspection (1). Fruit passing maturity tests are inspected to determine grade considering internal factors such as juiciness, dryness, firmness, flabbiness, sponginess, absence or presence of decay, freeze damage, injury, disease, etc. All fruit failing to pass either minimum juice content requirements or Brix to acid ratio maturity tests will be destroyed or legally diverted to some other suitable use. Maturity tests are also made on fruit for processing. These tests include juice content, acid, soluble solids, and soluble solids to acid ratio. In addition, wholesomeness is determined - wholesome fruit consisting of "fruit free from rot, decay, sponginess, soundness, leakage, staleness, or other conditions showing physical defects of the fruit". If after any required regrading, fruit is still found to be unwholesome or decomposed so as to be unfit for processing purposes, it will be destroyed

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at the expense of the owner, under the personal supervision of the inspector. For the fresh citrus fruit packer, no further quality tests are generally necessary. However, for the citrus processor, further flavor quality tests may be undertaken by quality control personnel at any of several points during production should a possible problem arise wherein flavor quality may have been adversely affected. The main emphasis, however, is made on the final product where generally a cross-section of each day's pack is checked for flavor quality either during the day of production or the next day (2). In Florida, USDA inspectors determine that flavor quality on all product meet explicit U.S. Standards and State of Florida Department of Citrus Statutes for Grades of the several types of citrus product packed. To maintain consistency in flavor grading, weekly panels are convened at the USDA Processed Products Branch Area Office in Winter Haven, FL to cover all citrus products (3). The primary citrus products evaluated are frozen concentrated orange juice (FCOJ), concentrated orange juice for manufacturing, orange juice from concentrate, pasteurized orange juice and grapefruit juice, the latter designation covering both "chilled" grapefruit juice and canned single-strength grapefruit juice. To obtain a U.S. Grade A classification for flavor of FCOJ, for example, standards state: "Frozen concentrated orange juice of which the reconstituted juice possesses a very good flavor may be given a score of 36 to 40 points. 'Very good flavor' means that the flavor is fine, distinct, and similar to that of fresh orange juice..." (4). There are also certain other factors associated with flavor grade, namely minimum and maximum degrees Brix to % acid ratio and oil content requirements. Should there be a question as to the flavor by either the inspector or the plant personnel, the inspector will submit a sample to the USDA Processed Products Branch area office for another evaluation.

In order to monitor the flavor of their products more closely, the Florida Citrus Processors Association has contracted with the USDA Processed Products Branch to operate a taste panel in which current FCOJ production is tested on a weekly basis. The panel consists of local consumers (generally housewives) and personnel from each of the participating plants (other types of citrus products have been evaluated in the past). Panelists rate samples on a 9-point scale from a low of "extremely poor" to a high of "excellent". Juices are ranked according to flavor score and a copy of the rankings are sent to each processor with only that particular processor's product identified (3).

A similar flavor survey is carried out at the University of Florida, Institute of Food and Agricultural Sciences, Agricultural Research and Education Center, Lake Alfred, FL (AREC). FCOJ, pasteurized orange juice, and orange juice from concentrate are examined on a monthly basis. Canned and bottled pasteurized single-strength grapefruit juices are checked biweekly. Sensory panels comprising 10-12 experienced taste panelists grade samples

on a 9-point hedonic scale. Unusual sample results are verified during further testing. Results of all samples examined each period are tabulated and sent to the participating plants, with only the hedonic score of the plant receiving the letter being identified, however.

In addition, a comprehensive sensory evaluation program involving citrus and citrus-based products is being conducted by the author. Many facets of citrus flavor research may be ongoing at any particular time utilizing an assortment of sensory evaluation methods.

In the United States and other countries, companies (primarily dairies) that reconstitute frozen concentrate for manufacture into single-strength products or manufacture FCOJ from bulk concentrate are also interested in monitoring flavor quality of their products.

Minimal attention will be devoted in this chapter to a review of the different types of sensory panels or to a discussion on operating and analyzing results of sensory panels. There are a number of excellent publications available describing and explaining nearly every facet of the aforementioned factors in detail (6-11).

This paper is an attempt to document certain problems peculiar to sensory evaluation of citrus fruit or products primarily as they pertain to Florida and to perhaps offer some solutions or alternatives to these problems.

I. Panelists

Special Criteria for Selecting Panelists. In addition to many of the usual criteria used in choosing taste panelists for sensory evaluation such as ability to differentiate bitter, sour, sweet, salty and bland basic flavor factors, determination of threshold values for these factors, proven ability to taste certain citrus juices, willingness to participate in taste panels on a regular basis, etc., there are a significant number of potential judges who cannot participate because of the chemical nature of citrus. For certain persons, for example, the acid present in citrus may affect them adversely, especially when the juice is not taken with a meal. Another possible problem is persons suffering from allergies to certain foods such as citrus. Fortunately, most consumers are very fond of oranges and orange products and there is not much of a problem enlisting individuals to participate in evaluation tests. However, grapefruit and grapefruit products are not nearly as universally accepted flavorwise, thus a special effort must be made to generate a grapefruit taste panel that will give consistent reliable results. Enlisting an individual who does not particularly like grapefruit on a grapefruit taste panel (perhaps even though the individual doesn't mind serving on the panel) can result in unreliable data. The individual will most likely expectorate the juice into an empty cup after only a cursory

examination, thus not allowing for a complete sensory evaluation of the product. Furthermore, since the product is distasteful to the panelist, proper motivation to do a good conscientious evaluation will be lacking, again resulting in unreliable data.

Inability of some potential panelists to taste certain flavor characteristics that may occur in citrus products at low or moderately low concentration levels may exempt these individuals from participating in routine survey, quality control, and perhaps certain other flavor evaluation work. For example, diacetyl in a citrus product imparts a buttermilk-like off-flavor. It may be detected in very small amounts by certain individuals, whereas the threshold for others may be significantly higher which would allow the presence of the diacetyl to go undetected. About two out of three otherwise qualified taste panelists cannot detect diacetyl in amounts that may be found in citrus products. Another compound, naringin, contributes significantly to the bitter sensation realized in grapefruit juice by most individuals, but not by all. Bitterness studies by Fellers (12) showed a remarkable spread in the ability of individuals to detect bitterness due to naringin. Two individuals were found who could not detect naringin concentrations at 2,000 ppm (or about five times the amount normally found in Florida grapefruit juice) in either model grapefruit juice systems or as an additive to otherwise pure grapefruit juice.

Number of Panelists. Very often practical considerations override the theoretical when it comes to the selection of the numbers of individuals to constitute an effective citrus taste panel. For several diverse reasons, many potential taste panelists either can not, will not, or would rather not participate in sensory evaluation panels. These individuals, along with those willing but found to lack sufficient taste acuity, can often result in only a few willing and able panelists at a particular location. Should this be the case, however, especially as pertains to discriminatory testing, numbers of individual evaluations may be increased through replication using the same panelists. Larmond (6) indicates a minimum number of panelists to be 4 or 5 to constitute a sensory panel, with laboratory panels usually being composed of 10 to 20 persons with 3 or 4 replications per judge per treatment.

At AREC, out of a pool of about 35 individuals available for everyday panel work, about half participate in orange product evaluation whereas slightly less than half participate with grapefruit. Panels consisting of about 12 semitrained and trained individuals are used. A 5 to 6 member efficient trained panel is generally available for certain sensory evaluation work. In addition, between 30 to 70 individuals can be utilized locally for small consumer-type panels. Large consumer tests often involve as many as 600 responses with data being obtained from several different demographic locations.

II. Sample Preparation

Fresh Citrus Juice. Freshly extracted commercial orange juice may contain a high peel oil content making sensory evaluation of this product very difficult because of the overriding, biting and even bitter flavor effect of the peel oil. This is one of the reasons why in certain plants, minimal attention is paid to the flavor quality of the juice until the product is standardized either in retail or bulk form (2).

Excessive pressure used in hand-reaming of citrus fruit can also result in juice containing high peel oil content and perhaps significant amounts of undesirable flavor constituents (primarily of a bitter nature) as well. Since high peel oil and undesirable flavor constituents would seriously affect sensory evaluation results in a negative way, care should be taken in hand-reaming citrus fruit to apply only as much pressure as necessary to extract most but not all of the juice. In this way the amounts of peel oil and undesirable flavor factors will be held to normal levels.

The number of fruit necessary to comprise a valid sample for flavor evaluation purposes may very well be in the range required by the Florida Department of Agriculture in determining citrus fruit maturity, namely 20 oranges, tangerines, tangelos, Temples or Murcotts, or 10 grapefruit (1). To test the validity of using a 10-grapefruit sample, fruit were utilized from an early season storage study involving 3 replicates for each of 3 treatments. Each replicate sample contained several boxes of fruit from the original lot of fruit. In all, 9 separate samples of 10 fruit each were taken at random from each of the replicate samples. The maturity indicators, degrees Brix, % acid, and the ratio between these two factors were determined on the juice of each sample. Juice was obtained by using a small electric hand-reamer that provided about 1,500 ml of juice per sample. The mean values for degrees Brix was 9.40 ± 0.125 (as determined by a table refractometer), 1.47 ± 0.03 percent acid (as determined by titration with NaOH), and 6.36 ± 0.125 degrees Brix to percent acid ratio (calculated). Since prior work by the author (13) has shown that a degrees Brix differential of about 1 in single-strength grapefruit or orange juice was required before differences could be detected by experienced judges, a difference of 0.25 degrees Brix between the highest and lowest samples, as found in the above study, would presumably not significantly affect flavor. In like manner, a ratio of degrees Brix to % acid of about 0.8 had been previously found to be differentiated in single-strength grapefruit juice using experienced judges. Again this amount was significantly higher than the 0.25 ratio found between the highest and lowest ratio samples in the above study, thus having an apparent negligible effect on flavor.

Citrus Sections or Segments. Sensory evaluation of this

type of product presents the problem of often significantly different flavored sections within and/or between fruit thus making it difficult for panelists to evaluate the overall flavor quality. Since the panelist will most likely sample only 1 or 2 individual sections or segments before passing judgment, no easy way exists to reduce the odds of maverick sections being tested with ambiguous judgments. An alternative method is to homogenize the product in a blender for as brief a period of time as possible to achieve a homogeneous mixture which may then be filtered through cheesecloth to produce a juice. Using this method, compensation can be made for any can-to-can variation by using several cans. Of course, texture cannot be taken into consideration using this procedure and, therefore, a possible significant part of the overall flavor may be compromised. If sections or segments are to be evaluated, it is a good idea to present the entire contents of a can, if practical, for each judge to make an overall evaluation. Seeds, if present, should be removed as their presence would only serve to confuse the panelist, especially if quality attributes are being evaluated such as firmness, sweetness, etc. Failure to remove seeds in this case could lead to a type of stimulus error, the seeds being an irrelevant characteristic.

For citrus sections or segments packed as chilled product either alone, as mixed types of citrus, or with other fruit such as cherries or pineapple chunks, there may be a problem for certain individuals tasting these products due to the presence of small amounts of sodium benzoate (0.05-0.06% w/w) which is added as a preservative. Sodium benzoate can impart a sharp flavor sensation to certain individuals even at the relatively low levels found in chilled citrus products. The sharpness of the benzoate flavor thus acts as an overriding factor, and the product will be graded down accordingly. Since individuals who have low tolerance to sodium benzoate will most likely not consume this particular product, or grade it down severely because of sodium benzoate, individuals chosen to evaluate chilled citrus products should have a relatively high tolerance to sodium benzoate.

FCOJ. Proper reconstitution of FCOJ is essential for valid taste panel results. Since orange juice flavor is of a rather delicate, mild, or light type, the water source used to reconstitute the concentrate must be relatively free of off-flavors, such as sulfurous. Distilled water should be used whenever practical. In addition, if the water used for reconstitution is placed in a refrigerator, walk-in cooler, etc. for chilling, care must be taken to have the water container covered securely to eliminate possible absorption of foreign flavors. And, of course, the reconstituted product itself, if being chilled prior to tasting, should be protected.

There are several methods available for reconstituting FCOJ so that the final desired dilution is correct. Reconstitution of concentrate requires use of a precise scale or balance, sugar

tables, and a precise refractometer. At times this precision may not be obtainable for sensory evaluation work when a good scale or balance or refractometer is unavailable. For reconstitution of retail products on a volume/volume basis, the following procedures or parts thereof may be followed: thaw frozen cans in running tap water (about 30 min for 6-oz cans); open can and place on a level surface and with a small knife or sharp instrument mark the inside of the can by making a short line at the surface of the concentrate; pour the contents of the can into a vessel large enough to contain all of the reconstituted juice; carefully fill the can to the mark with distilled water (chilled or room temperature, whichever is preferred) the prescribed number of times and add to the vessel; and stir very well until the concentrate and water are thoroughly mixed. Another possibility is to pour a certain amount of thoroughly thawed concentrate into a volumetric cylinder, carefully add the necessary volume of water and mix thoroughly. In a recent large scale consumer test of FCOJ in 3 U.S. cities (14) in which precise reconstitution Brixes of 12.8 and 11.8 degrees were required for about 1.5 l of each juice per session the following method was devised: make up cans of FCOJ containing exactly 6 fl. oz of product having a certain degrees Brix for the specific use in the test; at the point of testing place frozen cans in running tap water for 30 min; pour chilled distilled water into a 2,000 ml plastic volumetric cylinder until the water is exactly level with the top of the black mark (placed there in the laboratory previously marking exactly the number of ml or fl. oz required for exactly 12 fl. oz of FCOJ to make the desired final reconstituted Brix product); from the graduated cylinder, pour about one-third of the water into a clean mixing vessel; open two cans of FCOJ taking care not to incorporate water in the contents and not to spill the product; add the contents of the 2 cans to the chilled water in the mixing vessel; from the graduated cylinder pour water into each of the emptied cans to rinse the remaining product taking care not to overflow; add the rinsings carefully to the mixing vessel; pour all the remaining water from the graduated cylinder into the mixing vessel; and finally, stir or mix the contents of the mixing vessel very well making certain there is no unmixed concentrate on the bottom. This method in practice worked extremely well using premarked volumetric cylinders, two of which were available at each testing location (glass cylinders should not be considered in this situation because of the ease of breakage). Of course, identical reconstitution methodology could be applied to frozen concentrated grapefruit or other citrus juices as well as for FCOJ as discussed above.

The presence of surface or floating juice sacs may significantly affect sensory evaluation of citrus juices. Amount of floating pulp in any particular retail product is primarily dependent on corporate specifications as per their beliefs. When testing frozen concentrated citrus products containing signifi-

cant amounts of floating pulp, care must be exercised in the re-constitution process and in dissemination of juices for sensory evaluation work. If the entire can contents are to be used, there is no problem in reconstitution. However, if a half can is to be used for one treatment, for example, and the other half for another treatment, a thorough mixing of the thawed concentrate just prior to pouring will aid in obtaining equal distribution of the surface pulp in both samples. Again at the time of dispensing of juices to the individual cups, another thorough mixing will insure reasonably equal distribution of top pulp among the several cups. Of course, mixing will also insure good bottom pulp distribution as well. The above holds true for retail single-strength citrus juices as well.

Holding of Citrus Juices Prior to Sensory Evaluation. All types of citrus juices should undergo sensory evaluation in as short a period of time as is feasible following juice preparation because of the possibility of flavor change. For juice tested at room temperature, time between preparation and actual testing can be especially critical. Certainly, juice prepared one day for next day testing should not be considered even with overnight refrigeration. Generally, the most practical situation is morning preparation of juices for a morning sensory evaluation session and an afternoon preparation for an afternoon session.

Can-to-Can Variation. Experience has shown the variation in commercial products with identical codes for Florida product to be minimal. The small amount of variation that does occur is due to the nature of the manufacturing process which generally is not of the batch type. Production runs of from one to several hours duration, and including tremendous amounts of product, carry the same code with all of the variations of product existing, within certain plant specifications of course. However, plants having modern concentrate bulk-tank storage capacity in the 50,000-150,000 gal range (tank farms) and which utilize computer systems to meter out product for retail pack are in a position to be able to produce a more uniform product, perhaps, than a plant without such a capability. It has been the practice in retail FCOJ and chilled juice seasonal surveys at AREC to re-evaluate samples exhibiting any flavor quality problems. Occasionally the second or third cans tested will vary significantly in flavor quality from the original indicating real can-to-can variation. For all practical purposes, however, single cans of similarly coded product can be used in sensory evaluation work with a minimum amount of can-to-can variation to be expected.

III. Conducting Sensory Tests

Choosing a Sensory Test. The types of sensory methods available for citrus evaluation are numerous. Two excellent refer-

ences covering these methods in detail are the "Manual on Sensory Testing Methods" (5) and "Laboratory Methods for Sensory Evaluation of Food" (6). Selecting the right test for evaluating a citrus product as for other products depends primarily on what information is desired. Often several types of test may be available for answering the same question(s). It then may be a matter of trial and error to find the one test best suited for a particular situation. Occasionally, modifications of existing tests or newly designed tests are required in order to obtain certain information. Whatever test is chosen, statistical evaluation of the data should be made.

Experience has shown that differences between citrus samples can perhaps best be detected using the triangle test for orange, Temple, tangerine and allied products and paired comparison for grapefruit, K-early and similar products. Because of the inherent bitterness and relatively high acid in grapefruit, the amount of back and forth tasting required in the triangle test can result in significant loss of taste acuity during a test.

Utilization of a standard citrus juice product in a sensory method is not advisable in certain instances because of the tremendous amount of variability in the juices due to current commercial production practices. The primary variables that exist in the processing industry include: varietal differences, extractor and finisher variables, and variable usages of essence and/or essence oil, peel oil, and cutback juice. Certainly, a seasonal survey-type flavor study would be a poor place to employ a standard juice. However, a standard juice could perhaps be used to good advantage in a plant by quality-control personnel where variables would conceivably be reduced to a minimum. As more plants convert to tank-farm operations equipped with sophisticated computers, an end result will most surely be a more uniform or standardized product. Of course there are some basic problems in making standard-flavored citrus juices. However, using care, volumes of juices to be used as standard juices and having satisfactory flavor attributes could be produced each year and utilized from frozen storage as necessary in plants producing relatively standardized products.

Serving Vessels. For citrus juices both glass and paper serving containers may be successfully used. If glass containers are used, certain precautions should be taken to insure best results: (a) make sure that the glasses are thoroughly rinsed following washing to eliminate any residual soap or detergent off-flavors which might enter the citrus juice served next in the glasses; (b) for obvious reasons make sure that high standards of hygiene are practiced in washing the glasses by using clean, hot, soapy water, no washing of ash trays in the dishwasher, etc.; (c) use of ruby-red glasses aids in reducing any color bias and; (d) any glasses developing cracks or having chipped rims should be discarded immediately.

If paper cups are used, only the treated type should be employed as certain untreated cups can impart a "paper" or "card-board" flavor to the juice. Treated cups will also maintain their integrity longer. Use of certain marking pens to mark the cups should be avoided if it is noted that the ink imparts a lingering solvent aroma.

Volume of Juice to Serve. Experience has shown that about 1 1/2 to 2 oz of citrus juice is sufficient for a successful sensory judgment. Anything less than about 1 1/2 oz will very likely result in 1 or 2 individuals asking for more juice, especially if a test such as the triangle test is being used. However, it is important that however much juice is presented to the panelist, enough empty space is left in the glass or cup to allow at least a gentle swirling of the juice by the panelist in order for that individual to better detect aroma characteristics of the juice. Of course, in certain instances more than just 1 1/2 or 2 oz of juice may be desirable to serve panelists, such as for certain consumer-type taste panels or when a new product is being looked at; it then may be desirable for consumption of a full 4 or 6 oz serving, an amount such as an ordinary consumer might ingest at one time. Generally, only a single juice or perhaps two at the most should be considered per session with the larger volume of juice.

Number of Juices to Serve. Substantially more orange, tangerine, Temple, Murcott, etc. juice samples can effectively be served for sensory evaluation at one session than can grapefruit juices. The primary reasons for this are probably because of the notable amount of bitterness found in grapefruit juice and the relatively less acid associated with the orange and similar juices than grapefruit juice. Bitterness does not normally occur in Florida orange juice. However, bitterness can exist in orange juice under certain circumstances as discussed in the section in this chapter on bitterness.

As to actual numbers of juices that may be sampled at one session, a maximum of about 4-6 orange juices or 3-4 grapefruit juices are the practical limit for judges. We have found that anything much beyond these maximums severely tax the physical and mental capabilities of the average panelist resulting in a deterioration of reliability of results. In a consumer-type panel, probably, 1 or 2 juices per session would yield the best results. However, for a quality control person in a plant, USDA inspector, or other person involved with many evaluations, numerous orange and perhaps somewhat fewer grapefruit juice samples could be tested successfully with short breaks in between samples over several hours time. At AREC the author and two other experienced citrus sensory evaluators once successfully ranked by preference at one session as many as 16 different formulations of a new citrus juice product called "Sunshine-7" in which 7 types of

citrus were blended. Taste fatigue was evident in this arduous session but not so much as to significantly compromise the overall results.

Serving Temperature. For consumer or acceptance/preference testing, citrus juices should be served at a temperature in the range of 7-13°C or the approximate temperature at which juices are presumably consumed. However, for discrimination and perhaps certain other tests, testing at warmer temperatures in the approximate range 19-24°C may be superior to the lower temperature range. To test this hypothesis derived through experience, the first of several tests along these lines was recently completed (15). In this particular study, three different composite commercial FCOJs were evaluated over a 2-month period by an experienced 12-member taste panel, 3 times at 10°C and 3 times at 24°C, without giving the panelists any knowledge of the purpose of the testing either before or after each test. For 2 of the samples, mean flavor scores were found to be significantly higher at the 95% confidence level for the samples served at 10°C than those at 24°C. No significant difference was found in the remaining sample. Exactly why a particular citrus juice may be preferred chilled to unchilled by experienced panelists is not yet known. The cooler serving temperature could be the overriding factor. Another possibility might be in a suppression or masking of certain undesirable flavor notes by the chilled juice.

At whatever temperature citrus products are served, care must be exercised to make certain all samples have like serving temperatures. The least difference if detected by panelists will confuse them and result in questionable data.

Number of Types of Citrus Products Per Session. Should ever the occasion arise when more than one type of citrus product requires sensory evaluation, it is best to schedule a separate session for each product or group of like products. The human mind has a great tendency for automatic comparison and carryover of one experience to the next which in the case of sensory evaluation is not good should two or more dissimilar products be compared at one session.

In laboratory tests at AREC when a few fresh orange juice samples were included generally one at a time several times among many reconstituted FCOJ samples in a large survey study, the fresh juice samples graded out lower in flavor quality than the reconstituted FCOJ samples. However, when these same fresh juice samples were graded together as a group the scores increased.

What to Tell the Panelist. Panelists should be given as little information as possible about the test at hand so as not to influence results. Part of the same study as was described in the section dealing with sensory evaluation of more than one type of citrus product at one session also dealt with the problem at

hand. Results showed the fresh juice samples grading out well below the reconstituted FCOJ samples when no mention of the fresh juice sample was made. When several of the fresh juices were run together and the panelists were still not told what the product was, scores improved significantly. However, when the panelists were finally told that fresh orange juice was to be tested, the scores again improved measurably showing existence of a preconceived impression or expectation error.

A situation where panelists should be informed of the product they are evaluating is possible when certain new and/or quite different products are being tested. Two prime examples of this situation occurring at AREC involved flavor studies of an isotonic orange juice thirst quencher type of new product developed at the Lake Alfred Center, and bitterness studies utilizing model grapefruit juice formulations. Some panelists were quite unforgiving when not told beforehand what it was they were about to evaluate. For discrimination testing, no harm should result from product identity being disclosed.

Another factor to consider is whether or not to have a list of flavor attributes available for the taste panelist to peruse while judging the product. Perhaps in certain special instances, such as when specific flavor attributes or intensity of attributes are being considered, a listing may be used. However, normally no such listing should be given allowing each panelist to judge the product "from scratch" with a minimum of preconceived impressions entering in. Also, no matter how many items might appear on a listing of flavor attributes (and there could easily be as many as 20 for citrus products), the occasional odd flavor will appear and the panelist should feel absolutely free to describe it without the presence of distracting data.

Value of Soliciting Comments. Very often number data that is collected through sensory evaluation, whether found significant or not, can be explained fully or at least partially through examination of panelists' comments which were also obtained at the time of testing. Every sensory evaluation test should have space for comments. This is both for the psychological advantage it gives the panelists to express their feelings on some factor that perhaps did not or could not be included as part of the test and, also, to better understand how/why the panelists answered the way they did. It is possible at times to utilize frequency of individual comments and/or total comments for samples in statistical evaluation of data, thus possibly gaining additional information from the test.

Value of Using a Regular, Trained or Experienced Sensory Panel. An advantage in having this type of panel is the facilitation of interpretation and understanding of each panelist's evaluation it makes for the supervisor. In time an alert panel supervisor will know and understand individual preferences, dis-

likes, and idiosyncrasies and thus, make a better evaluation of the data gleaned from the sensory test. For example, one capable panelist at AREC possesses excellent aroma and taste acuity for the presence of diacetyl in citrus products; however, unlike the other panelists, does not particularly object to the presence of this "off-flavor" in citrus products. A similar situation can exist if bitterness is detected in orange juice by certain capable panelists. Bitterness normally is considered highly undesirable in orange juice, however, the occasional individual able to detect bitterness does not consider it especially undesirable and will fail to rate the juice down significantly. Each individual has his or her own ranking for relative importance of flavor attributes constituting good juice and bad juice.

The alert panel supervisor will also come to know about each regular panelist's ability to detect the many off-flavors that could possibly occur in citrus products. Of primary concern, other than the common sweet and sour flavor attributes, are the panelists' abilities to specifically identify: (a) bitterness (naringin and/or limonin induced), (b) heated, processed or pump-out off-flavor, (c) excess peel oily, (d) excess and/or poor essence flavor, and (e) the following off-flavors: cardboard, tallowy, castor oil, diacetyl or buttermilk, green or immature, overmature or stale fruit, and spoiled fruit.

IV. Varietal Considerations

In Florida the range of flavors in orange juice products due to varietal differences can be very wide. Each mature major Florida variety possesses a general flavor profile as follows: Hamlin (early season) - moderate orange flavor with no top notes; Pineapple (midseason) - moderate to full orange flavor with a fruity top note; Valencia (late season) - full orange flavor with good body; Citrus reticulata, (or hybrids thereof), such as tangerines, Temples and tangelos possess distinct flavor top notes quite unlike the 3 major orange varieties. For this reason in the manufacture of FCOJ, for example, only up to 10% by volume of the concentrated blend of juice can be of the Citrus reticulata type (4). The same standards limit Citrus aurantium (sour orange) to 5% by volume.

The major Florida grapefruit varieties are the nearly seedless Marsh white and allied pigmented forms, such as Marsh Pink, Thompson and Ruby Red, and the seedy Duncan. Differences in flavor between the nearly seedless and seedy varieties are not large, however, the consensus of the author and others familiar with both types is that seedy fruit possess a somewhat fuller, more balanced grapefruit flavor than seedless.

Extreme care must be exercised when applying general flavor terminology to orange, mandarin or grapefruit types of citrus when there are so many varietal flavor differences within the basic types.

Of course there are many other factors involved in flavor of individual varieties of citrus such as seasonal variation, time of harvest or maturity, soil type, rootstock, geographical considerations, etc.

V. Color Effect

A large consumer sensory test conducted at the 1965 World's Fair in New York (16) involving several different orange juices of varying quality attributes, including color, showed that color was one of the most influential factors in juice preference. In a recent study by Huggart et al. (17), grapefruit juices were prepared having varying levels of visual color from white through pink, with all other factors being equal. A large consumer test of the 5 products showed color to have a significant effect in acceptance of paired juices. In general, the yellowish-white to brownish-yellow (chamois) juices were preferred over either white or pink juices.

With there being color biases/preferences in both orange and grapefruit juices, care must be exercised in sensory evaluation work to insure against these color biases. An effective means of neutralizing any color bias in citrus sensory evaluation work has been found to be the utilization of red lights in the booths used in making the evaluation.

A special situation can exist in Florida and other citrus producing areas relative to color bias in orange juice. Individuals working with citrus are familiar with the different varieties and have developed their opinions as to relative quality. Hamlin orange juices, for example, are considered by many to be the least preferred of the major varieties whereas Valencia generally passes as the most preferred. Color of Hamlin juice is light orange whereas Valencia juice is dark orange, thus should panelists who are familiar with the flavor of different orange varieties be able to see the color of the orange juices being evaluated, a possible bias due to color might exist. Again, the use of red lights in the panel area to neutralize product color should be utilized.

VI. Specific Citrus Flavor Attributes

Bitterness Considerations. Bitterness is not normally found in commercial Florida orange juice, but when it does occur, the source of the bitterness is difficult to determine from a sensory evaluation standpoint. Bitterness, primarily from limonin (18), may be due to one or more of the following: too severe juice extraction, use of certain Brazilian juice (19), use of Navel oranges, excessive peel oil probably greater than 0.020% by volume, and perhaps other factors as well.

Orange pulpwash (water extraction of soluble fruit solids from orange pulp) can contribute significantly to the bitterness

and harshness (roughness) of orange juice to which it has been added (20). U.S. standards for grades of FCOJ (4) state "In its preparation, seeds, (except for embryonic seeds and small fragments of seeds that cannot be separated by good manufacturing practice) and excess pulp are removed, and a properly prepared water extract of the excess pulp so removed may be added." Florida, however, does not allow pulpwash in FCOJ (1). Orange pulpwash is allowed in concentrated orange juice for manufacturing "if product is not to be used in the production of FCOJ in retail or institutional sized containers, it may contain soluble orange juice solids recovered by aqueous extraction or washing of pulp removed from the juice contained in the product, but not from pulp removed from other juice" (21). And concentrated orange juice for manufacturing can be used in the manufacture of orange juice from concentrate (22) and as 25% of the total orange juice solids in finished pasteurized orange juice (23). A study is currently underway to determine how widespread is the use of orange pulpwash outside Florida in FCOJ and orange juice from concentrate and how much pulpwash has been used in each of the products (20). Sensory evaluation of each product is also being carried out along with the other analyses.

Grapefruit contain the bitter principles limonin and naringin and these give grapefruit products the characteristic "bite" for which the fruit is noted. In an effort to understand more about consumer likes and dislikes of grapefruit and grapefruit products, a part of the flavor research at AREC has focused on the bitterness attribute. In conducting this research, and some other, a few rather unique problems arose that will be considered here. From a technical point of view, there are extreme difficulties in getting water-insoluble limonin into aqueous mixtures, such as citrus juices or model citrus juices, without addition of an unwanted solvent such as alcohol. Naringin may be added to citrus juice or model citrus juices without heating the products using the following method: starting with high Brix citrus concentrate, calculate the amount of water necessary to achieve the desired final lower degrees Brix concentrate or reconstituted juice. Determine the amount of naringin to add to achieve desired ppm in the final product. Heat a portion of the water to about 65°C and add the naringin while stirring. Bring the water/naringin solution to room temperature and add to the concentrate or reconstituted juice. Add any remaining required water. This method will work up to at least 2,000 ppm naringin in the final reconstituted juice. Concentrate treated thusly may be frozen for later use. Care must be taken, however, not to chill any single-strength juice having added naringin as precipitation will occur. One method used by Fellers and Carter (24) to incorporate natural bitterness into grapefruit juice was to add certain percentages of a frozen concentrated grapefruit juice pulpwash obtained from use of "hard-squeeze" fruit.

Reference has already been made in the section dealing with

"Special Criteria for Selecting Panelists" on the inability of certain individuals to detect naringin at concentrations as high as 2,000 ppm in aqueous model grapefruit juice systems or grapefruit juice. Obviously those panelists would be of no value in discriminatory sensory work involving naringin.

In a recent large consumer test (25) in which naringin was added to five like portions of a good frozen concentrated grapefruit juice in increasing quantities up to about 2,000 ppm in the reconstituted juice, a key finding was that generally, as the level of naringin increased, perceived bitterness and tartness or sourness increased and perceived sweetness decreased. The primary ramification of this finding is that extreme care must be used when interpreting sensory evaluation data on fresh and processed grapefruit juice products as regards bitterness, sweetness and sourness.

Occasionally grapefruit juice samples can be excessively bitter, and can cause problems for panelists involved in evaluating juices having these occasional juices mixed in. The problem is that the intense bitterness can render one's sensory acuity nearly useless for a significant period of time resulting in possible erroneous data for succeeding samples, even with ingestion of a cracker and/or water. According to Dougherty and Barros (26) it is possible to have greater than 1,000 ppm naringin and/or more than 10 ppm limonin in certain commercial grapefruit samples. However, to have both maxima occur together would be indeed rare. Of course, randomization of samples should work to equalize any contrast effect caused by the bitter sample.

Heated, Processed or Pumpout Flavor. Prior to the advent of the temperature-accelerated, short-time evaporator (TASTE), commercial FCOJ and frozen concentrated grapefruit juice samples often possessed an undesirable heated, processed or pumpout flavor that was not masked entirely by oil, cutback, or essence addition. Current manufacturing practices have effectively eliminated this problem in the products. However, pasteurized orange juice, orange juice from concentrate, canned orange juice and all their grapefruit juice counterparts possess a wide range of heated or processed flavor, from a negligible amount to extreme. As to how this heated flavor is evaluated apparently differs from person to person and from group to group. According to U. S. standards for grades of orange juice from concentrate (21), for example, for this product to make U.S. Grade A it "possesses a very good flavor" when "very good flavor means that the flavor is fine, distinct, and substantially typical of orange juice extracted from fresh, mature sweet oranges; is free from off-flavors of any kind;etc". Since a heated or processed flavor is considered to be an off-flavor and exists in most commercial orange juice from concentrate, apparently the U.S. standards do not take this flavor into account. It is interesting to note that for proposed U.S. standards for grades of grapefruit juice (27), that in the pro-

posed flavor definitions for both grapefruit juice and grapefruit juice from concentrate, that "The grapefruit juice may be slightly affected by processing, packaging or storage conditions."

Apparently like so many other flavor attributes of citrus products, heated, processed or pumpout type of off-flavors vary considerably in individual threshold and tolerance levels. The author, for example, possesses both a low threshold and tolerance for this particular off-flavor in any type of citrus product.

Cardboard, Citrus Oxidized, Castor Oil, Tallowy and COF Off-Flavor. For this group of off-flavors there exists much confusion. Olsen *et al.* (28) believed these off-flavors were the result of oxidation of substances in orange concentrate since they were able to produce these off-flavors in concentrate by whipping air into the product. Blair *et al.* (29) in their studies referred to these off-flavors as the "COF effect", COF being an abbreviation for either citrus off-flavor, citrus oxidized flavor, or castor oil flavor. Actually all of these off-flavors may indeed be allied as being products of oxidation, however, in most instances juices so affected may be characterized by the experienced individual as possessing either cardboard, castor oil, or tallowy off-flavor quite distinctly. Should none of the above apply then usage of the more general term citrus oxidized flavor could be used. Vague terminology such as COF should not be used and can only serve to confuse this particular flavor situation.

Essence Considerations. Participation in survey-type sensory evaluation panels of Florida FCOJ, orange juice from concentrate, pasteurized orange juice, and grapefruit from concentrate at AREC for the past 15 years has resulted in many observations by the author as to the dynamic flavor picture presented to the consumer by the citrus industry. Perhaps one of the major changes noted has been the marked increase in the use of essence and essence oil in recent years, notably in the orange juice products. No figures are available on amount of FCOJ packed in Florida containing essence/essence oil but a figure between 50 and 75% may not be unrealistic. Prior to essence usage as a flavor-enhancing material, cutback (freshly extracted orange juice), cold-pressed orange oil, folded oils or combinations of the above were the prime flavor enhancement materials. The addition of cutback juice to the concentrated juice from the evaporator was the key to the invention of FCOJ (30) in 1948 and is still being used today by a significant number of Florida processors.

The major problem in sensory evaluation of citrus products containing essence appears to lie mainly in individual preferences as regards essence-add products and individual thresholds for the essence fraction in these products. A significant number of regular, trained panelists at AREC, for example, upon detection of certain essences in citrus products might grade the pro-

duct down and comment that the product is "perfumey", "artificial" or "synthetic" flavored. Others, also detecting the presence of essence, might grade the product up and comment "tastes like fresh juice" or something similar. And then there are those individuals who apparently are not affected one way or the other by essence addition.

Dougherty *et al.* (31) found that orange essences produced by different recovery systems were distinct and differed considerably in their chemical composition and strength; in some cases there was an optimum level of essence that could be added to FCOJ that would produce a better flavored juice; that higher levels of essence could result in a lowering of flavor quality; and that essence-enhanced FCOJs did not lose significant amounts of essence flavor over a 30-month frozen storage period. Results of this study showed some of the reasons why there were often such a disparity of grades and comments when essence-bearing FCOJs were being evaluated (especially in survey-type flavor studies). Not only were the FCOJs anything but standard products due to numerous factors (many of which have been discussed elsewhere in this chapter), but the essences used in these products also proved to be anything but standardized resulting in a mixed bag of flavor sensations.

Serving temperature has not been shown to affect flavor in essence-bearing citrus products. However, if temperature was found to be or suspected of being a significant factor, serving temperature of samples destined for preference-type sensory evaluation should be made at temperatures at which the product would normally be consumed (presumably about 7-13°C).

A final consideration concerning essence in citrus products is that depending on the type and/or quantity of essence used, detection may be by aroma, taste or by both. Proper sensory evaluation of all citrus products should incorporate both aroma and taste judgments - a taste judgment alone just not being enough in many instances. Should, for example, a sample containing poor essence, "wet dog" essence, "buttermilk" essence, etc. occur primarily in the aroma fraction, a judgment based on taste alone might miss the off-aroma.

VII. Problems in Citrus Sensory Evaluation Research

Avoiding Flavor Highlights in Control Samples. Every effort should be made in utilizing a characteristic type of citrus product when conducting certain sensory evaluation studies, especially if studying one or more specific quality attributes. The following are the major flavor highlights that should be avoided: excessive cold-pressed oil (greater than about 0.017% (v/v) in orange; 0.010% (v/v) in grapefruit), moderate or high essence product, orange product having any detectable bitterness or grapefruit having excessive bitterness (greater than about 4 ppm limonin or 500 ppm naringin), degrees Brix to % acid ratios nei-

ther too low nor too high thus keeping product neither too acid nor too sweet, too thin or watery, excessive top pulp, and no hint of any of the following off-flavors: cardboard, castor oil, tal-lowy, diacetyl (buttermilk), green or immature, overmature or stale, or spoiled or rotten fruit; in short nothing unusual about the aroma or taste that might confuse or distract the panelist.

Use of Model Systems. From time to time it may be desirable to utilize a model or artificial citrus juice system as an aid in studying certain flavor attributes. A big advantage, of course, is the resultant standard "juice" being completely reproducible at any time. However, the big disadvantage is that no matter how well a citrus product is simulated in a model juice system, many taste panelists apparently cannot feel really comfortable when evaluating a purely artificial product.

A basic formulation for making a model or artificial grapefruit juice, for example, might include sucrose (sweetness), citric acid (sourness), potassium citrate (buffer), pectin or alginate (body), naringin (bitterness), and cold-pressed grapefruit oil emulsion (grapefruit flavor). Of course, use of additional ingredients to further refine the product could be added. To achieve an oil emulsion suitable to add to a product such as described above, the following formulation may be used (for an approximate 10% emulsion, for example, to produce about 200 ml): mash 5g gum acacia and 50 g cerelose to a fine powder and mix; add 20 ml of good cold-pressed grapefruit oil to the mix and stir well; measure out 125 ml water and add just enough to the mix to form a slurry; pour into a blender using some more of the measured water to rinse the container; mix the slurry using the blender for a short period of time; pour the slurry into a container using the remainder of the water to rinse the blender; close the container tightly and keep refrigerated until ready to use. Approximate usage levels for such a flavor emulsion might be 4 drops/100 ml of model or artificial juice.

Isotonic or High Energy Thirst-Quencher Type Citrus Products. Several years ago many formulations of this type orange juice product were successfully made at AREC (32). Flavor studies associated with this work by the author were complicated by the fact that the energy drink product was designed for active people to quench thirst and restore energy. So that even though basic sensory evaluation work could be accomplished by panelists in a booth, the products had to be thoroughly field tested, which they were, using local high school and college football teams primarily. The active field trials, for example, showed conclusively that pulp levels had to be lowered significantly - a factor not considered important during the static evaluations.

The importance of testing new or improved citrus products and where and how they will ultimately be consumed cannot be overly stressed. The success rate for new products is poor

enough without a product being introduced without having had a thorough research and development program carried out on it, including trial consumer tests.

During the testing of the many formulations of the thirst quencher product, another problem arose - that of excessive time being required for storage tests of the products packed as single-strength juices in glass and cans. The answer here was accelerated storage tests utilizing a large 43°C insulated box. Using this system months of storage at room temperature (20-22°C) were condensed to days or at most weeks with sampling periods running every 2-3 days for sensory evaluation.

Geographic Considerations. Occasionally results of decisions made at AREC relative to new products or certain flavor attributes are found to differ when checked elsewhere, exhibiting certain geographic differences in tastes or preferences. For example, in two different situations (one of which is described (33)), minimum bitterness levels were set in Florida following both small trained panel tests and larger consumer tests to definitely include levels found to result in unacceptable overall flavor in grapefruit juice products. The same samples, however, were rated significantly higher outside Florida. Perhaps Central Floridians are more aware of the tremendous range in flavor of grapefruit harvested over the season and naturally tend to rate down a product exhibiting what they feel and know to be an inferior flavored juice.

A point to consider with fresh fruit flavor is that a lot of fresh fruit, especially oranges, found to possess fresh, clean flavor in a citrus producing area may be anything but that following long periods of transit and storage outside of that area. Unlike many other fruits and vegetables, citrus fruit never increase in flavor quality following harvest - only decrease, the rate depending on many variables. Any sensory evaluation work carried on with old fruit, whether in or out of the citrus producing area most likely would possess a significant amount of stale flavor resulting in possible erroneous or variable data, especially if the supposed "fresh" juice is being used in research work.

Testing of Potentially Hazardous Compounds. A possible danger exists in testing the effect on flavor of federally non-cleared substances that may enter into citrus products. A sensory evaluation supervisor has a clear obligation to refrain from allowing unknown potentially hazardous substances in his or her research.

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Immunological Tests for the Evaluation of Citrus Quality

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Quality of food products is nearly always based on esthetic values, such as color, texture and consistency, as well as physiological thresholds and sensitivities to taste, flavor and aroma. Ultimately the acceptance of foods and their products is dependent upon the physiological or biochemical state of the product, however, the subjective evaluations cannot be ignored except in times of hunger and famine.

Each food and food product is, then, a veritable collection of chemical constituents which is neither static nor stable. It might be best to describe the endogenous chemistry as dynamic and, as such, the quality characteristics must be a function of the composition of the chemical constituents at any given time. Since the basis of chemical composition is, in fact, controlled by the inherent genetics of any given organism, we can at least focus in on the fundamental site responsible for each chemical compound. In addition, however, we must also be aware that the interactions of these same genes with a multitude of complex regulatory mechanisms also plays a major role in determining the ultimate chemical composition at any given period of development.

Investigation into the chemical composition of citrus has led to the isolation and identification of hundreds of individual compounds. As work has continued, biochemical, nutritional and organoleptic studies have made great strides in determining which compounds are paramount in the final determination of fruit and fruit product quality. For the purpose of this discussion, we will concern ourselves with only those quality aspects affected by endogenous chemical composition and consideration of physical or descriptive parameters will be omitted.

Food Quality

Quality is defined as "the nature, excellence or intrinsic level of a thing" and thus evaluation is a complicated and difficult task. The major problem related to quality evaluation is inherent in the methodology used to make the determination.

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The most obvious method is subjective and here the evaluation is based on the opinion of the tester and the response of the sensory organs. Obviously this technique presents many problems and there is wide variation in the range of results obtained. Objective methods are based on specific analyses and, thereby, exclude tester opinion. Thus, the intrinsic aspects of quality are reduced to chemical, physical, or biological measurements and only the interpretation of the resulting data is related back to the subjective opinion.

Reduction of the subjective values or criteria to a specified compound, group of compounds or physical factor is often a very difficult endeavor, but one by one each of the so-called "qualitative criteria" is being linked to the presence or absence of specific constituents. Sometimes only a single compound is involved, however, often the qualitative character is the result of additive or even synergistic interactions of two or more constituents. Thus, since there are so many different characteristics that can contribute to the total quality of any single food product, it is paramount that precise objective methodology be developed for each parameter.

In citrus fruits and products, most flavors and aromas are produced by polyphenolic compounds or essential oils plus a variety of non-volatile organic compounds. For detailed discussions of citrus flavors and chemical composition, the reader is referred to the excellent and comprehensive reviews which have been published (1,2,3,4).

During the past half-century, the citrus industry has grown world-wide and quality standards, both local and international, have continually become more comprehensive and rigorous. Numerous definitions and criteria of quality have been published (5,6,7) and in some areas, e.g. Florida, wide ranging guidelines have been adopted and laws enacted for the sole purpose of maintaining quality levels. Extensive advances have been made in quality control or quality assurance programs (8) yet many difficult and complex problems remain as intrinsic components of the industry. Thus as the impetus for quality improvement has evolved, it has become a widely recognized fact that certain technological advances were required before qualitative progress could be realized.

The purpose of quality control or quality assurance in the citrus industry is to help provide for the production of a uniform, high quality and commercially acceptable product. For most of the major qualitative characteristics, standardized, accurate and simple methods have been developed, and high and low limits have been carefully been established. Some of these include: Brix, acid, color, recoverable oil, and free and suspended pulp. For each of these criteria, a simple and rapid assay has been developed and good correlation exists between the objective assay results and the subjective quality evaluations.

Problem Areas in Citrus Quality

In spite of the progress which has already been made, there still exist, in citrus fruit and products, numerous qualitative aspects which are either very difficult to measure or whose contribution to quality is a function of the organoleptic sensitivities of the consumer. For the purpose of this paper we would like to concentrate on two groups of naturally occurring compounds which have a major impact on the taste of citrus fruit and their products.

As previously discussed by Maier and co-workers (Chapter 4), in Navel, Shamouti and certain other orange cultivars, the presence of limonin, a bitter triterpenoid, causes many economic and organoleptic problems and greatly affects the taste quality of processed fruit. Limonin is also prevalent in the grapefruit but the intrinsic quality of this fruit is further complicated by the presence of naringin, a bitter flavanone neohesperidoside (Chapter 5).

Numerous organoleptic studies have been done on bitterness as a function of limonin and/or naringin, and a myriad of additive and perhaps synergistic results have been obtained (9). Table I presents a generalized response as measured in a variety of taste studies.

Table I

Organoleptic Responses to Limonin and Naringin

	<u>Limonin</u> ppm	<u>Naringin</u> ppm
Non-bitter	6	20
Slightly bitter	7-9	20-300
Bitter	10-16	500-1300
Very bitter	18	1500
Threshold	ave.=1	ave.=20

However: a solution or juice with 0.75 ppm limonin and 5 ppm naringin is bitter (99% confidence level) (9)

During the past two decades interest and concern about the bitter principles of citrus products have increased greatly and although some excellent chemical studies have been done, there

has been little information generated which has had an effect on fruit quality. The major problem which has proved most difficult to overcome in these studies is that unlike other quality factors, e.g. sugar, acid, etc., the bitter principles cannot be rapidly assayed and quantified.

Current Analytical Methods

When one examines the analytical methods which are presently available for these bitter compounds, it is clear why the study and control of bitterness in citrus has been so hampered and quality control virtually lacking. For the flavanone, naringin, only an approximate test is available (Davis Test) (see Chapter 5 for a discussion of this method) and for limonin there is no method available for monitoring in processing plants.

In the past ten years thinlayer chromatography (TLC) has proven useful for semi-quantitative measurements of fruit and juice samples, however this method is by its very nature: 1) rather insensitive (μg range); 2) slow (1 hour or more for sample application and solvent development); 3) requires pre-purification steps (liquid-liquid extraction or precipitation); 4) detection is difficult (especially true for limonoids) and 5) sample throughput is normally less than 50 per person per day.

Within the past several years good separation and quantification have been achieved with high-performance liquid chromatography (HPLC) and this procedure has proven very useful. However, HPLC is not without its drawbacks: 1) equipment cost is very high (thus small processing operations would probably be unable to purchase and maintain such an instrument); 2) only one compound can be measured at a time since different columns and solvents are used for each class of compounds (this also means that time must be spent in changing the system from one analysis to the next); 3) pre-purification is required and for good resolution repeated liquid-liquid and evaporation steps are involved; 4) the procedure is slow (only 10-15 samples can be processed per person per day); 5) it is sensitive only to the parts per million (ppm) range ($\mu\text{g}/\text{gm}$). Thus, in summing the current status of limonin and naringin quantification, a quotation is most appropriate.

"None of the methods developed thus far can be considered ideal, especially for routine quality control purposes. Among their disadvantages are subjective readout methods, time-consuming separations, questionable specificity, and limited applicability. Recent work showing that taste thresholds for limonin are lower than had previously been assumed for significant proportions of the population emphasizes the need for a better assay method. The use of a protein specific for limonin, such as an enzyme or antibody, could provide the basis for an improved assay." (9)

Development of the Immunoassay in Plant Science

As we began to develop interest in the bitterness problem in citrus fruit, a major advance or breakthrough in plant science was achieved when it was shown that an immunological assay method could be developed for the measurement of naturally occurring plant products (10). In this study it was demonstrated that it was possible to develop antibodies which were specific for the compound under investigation, and the authors furthermore suggested that this immunological method should render itself exceedingly useful in plant screening and breeding programs; in cell culture screening; and for studies of biosynthesis, transport and metabolism. Extension of such versatility would logically include quality control studies and monitoring.

The role of the immunoassay, especially the radioimmunoassay (RIA), in clinical biochemistry has been the major factor in the tremendous advances made in that field since its introduction in 1959 (11). At present the RIA is the most powerful analytical tool available for quantitative detection of molecules of diverse structure and function in biological fluids of human, animal and now plant origin. The immunoassay comprises a unique combination of sensitivity and specificity as well as precision and applicability. With this assay technique, it is now possible to detect and very accurately measure compounds at endogenous physiological concentrations which frequently are in the range of 10^{-12} M or lower. In Table II the major characteristics of the immunoassay are listed. This method is versatile, specific, can be utilized for almost an unlimited number of compounds and has a high throughput potential.

Table II

Characteristics of the Immunoassay System

- 1) Most sensitive analytical technique for both routine and sophisticated analyses.
picogram (femtomole) range = parts
per billion range = cellular level
- 2) Specificity - nearly absolute for each compound permitting direct measurement in crude extracts, nutrient solutions or processed samples
- 3) Suitable for automation or semi-automation. Rapid analysis possible, 20-30 minutes.

- 4) Can be used for single sample analysis or for mass screening. Screening potential is as high as 3000 samples per day per person.
- 5) Useful as a quantitative or semi-quantitative assay

The role of the immunoassay in food science has developed more slowly and, overall, rather few immunological tests are currently being performed on a routine basis. Table III provides a list of some of the major analyses which are available (12).

Table III

Immunoassay in Food Science

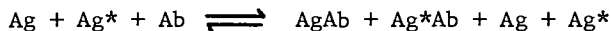
- A. Food Proteins
 - 1) meat
 - 2) soybean, wheat, barley, peanuts
- B. Toxic Plant & Animal Constituents
 - 1) agglutinins
 - 2) proteinase inhibitors
- C. Bacterial and Viral Toxins
 - 1) enterotoxins from Staphylococcus, E. coli, Clostridium
 - 2) fish and shellfish poisons
 - 3) foodborne viruses
- D. Food Allergy
 - 1) allergens
- E. Citrus (discussed below)
 - 1) Estimation of orange juice content in soft drinks
 - 2) Bitter principle analysis (limonin and naringin)

Within the last several years there has been a very rapid expansion of the RIA into plant science, and assays are presently in use with such diverse compounds as nicotine (13), indolacetic acid (14), abscissic acid (15), serpentine (16), digoxin (10,17) and many others. The unique features of the RIA have shown that it is an excellent and very necessary technique for mass screening programs involving genetic breeding of plant characters (18,19) screening of cell cultures for primary or secondary metabolites (20), for investigations of bio-synthesis (21) and in practical situations such as quality control monitoring of plant extracts (20).

The immunoassay itself can be performed in a wide array of procedural variations, but the RIA is presently the most widely used. In principle, the immunoassay is based on the highly specific reaction of antibodies with antigens against which the antibodies have been directed. The majority of antigens known to biology are proteins or other molecules of high molecular weight. Compounds with a molecular weight below 1000 are usually not

immunogenic when introduced into the bloodstream of an animal. However, if these low molecular weight compounds are bound covalently to protein carriers (such as human serum albumin or poly-lysine) as haptens they become immunogenic and specific antibodies against these haptens are produced.

The production of antibodies is normally done in rabbits and it typically takes a minimum of three months, with booster injections, to produce a final antiserum with a high titre. Characterization of the antiserum is done by the following: a) determination of the titre, (this is defined as that dilution of the antiserum which binds 50% of a known amount of radioactive or labelled hapten under constant assay conditions). In most cases the label is ^{125}I or ^3H but non-radioactive immunoassays are also available in which an enzyme or fluorescent compound functions as label, b) determination of the effects of pH, solvents and chemicals, e.g. ethanol, azide, etc.; c) correlation of the immunoassay with standard methods; d) determination of specificity. This last characterization factor is extremely important since compounds other than the hapten might bind with the antibody. This phenomenon is known as cross reactivity and a wide variety of known related compounds must be tested. After production of the antiserum and its characterization, a single animal should produce enough material to be used in tens of thousands of single assays. The principle of the RIA is diagrammed below and the assay is done as follows:



where: Ab = antibody

Ag = antigen

Ag* = antigen labelled with ^{125}I , ^3H , enzyme, etc.

To a solution of known titre antiserum is added a known concentration of radioactive or tracer hapten (antigen) and an aliquot of the plant extract. There will be a competition of the labelled hapten (added) and unlabelled (plant extract) hapten for the fixed number of antibody sites (antiserum of known titre) and this results in some of the labelled hapten being bound while the remainder remains free. The distribution of the radioactive hapten between the free and bound state will be a function of the amount of unlabelled hapten present in the assay tube. After equilibrium has been reached, the bound and unbound hapten can be separated (Fig. 1) (several methods are available) and determination of the radioactivity in either fraction gives an exact measure of the unknown hapten (plant extract) as computed from a standard curve (Fig. 2).

Since the immunoassay should prove to have great potential in all areas of plant science, we would like to describe in some

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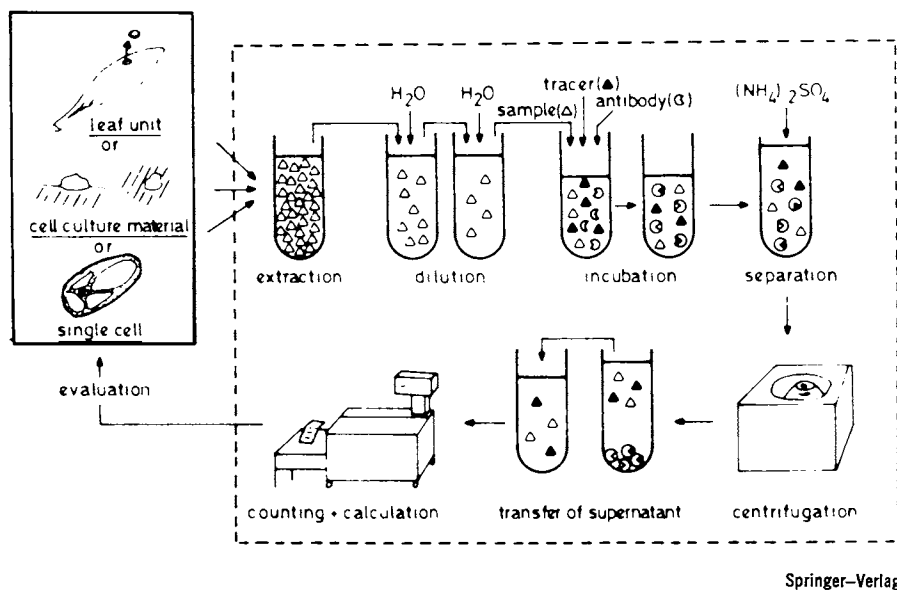
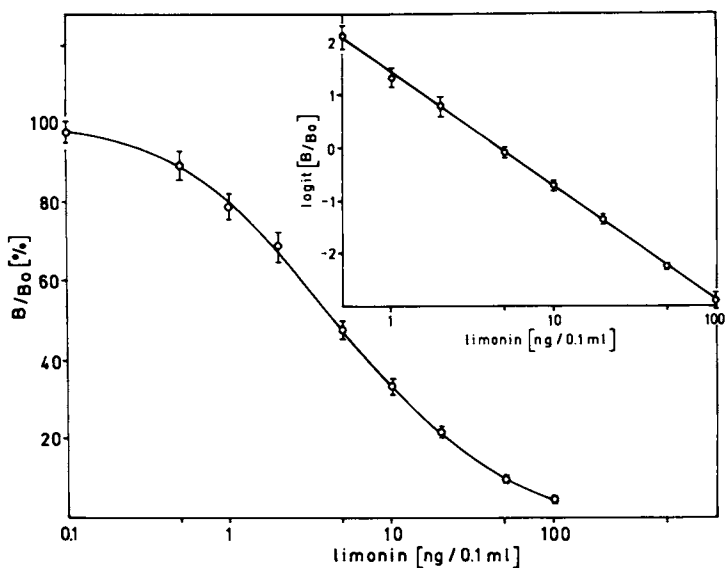


Figure 1. Performance of the semiautomated RIA technique (36)



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Figure 2. Limonin standard curve shown in two different plots (—O— Mean \pm s.d. of triplicate determinations; tracer is ^3H limonin (Sp. Act. 22 Ci/mmol)) (23)

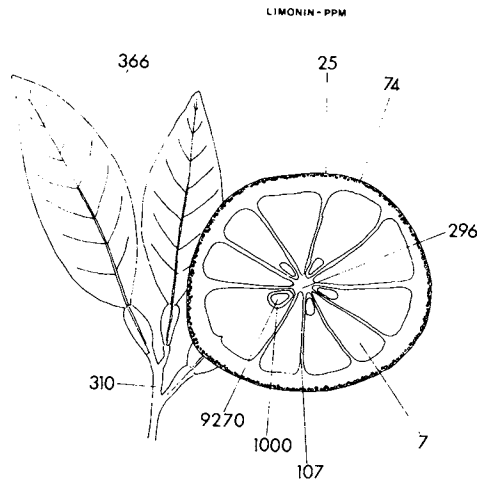
detail results of studies which we have done using limonin as the antigen. During the past two years we have developed and characterized two different RIA's for limonin (22,23). One RIA was done using ^{125}I -limonin as tracer and the antibody developed is characterized by having a high affinity ($k_d = 1.1 \times 10^7$ l/mol) for limonin. The detection limit of the assay is 0.07 ng per 0.1 ml or 0.7 parts per billion (ppb) and the standard curves are linear over a range of 0.5-100 ng limonin. Assays can be performed in crude extracts and 400-800 samples can be processed per day by one person. Coefficients of variation of triplicate B/Bo values throughout the measuring range were $2.5 \pm 1.8\%$ and recovery values were found to be 93%. The antibody produced against limonin has a very high titre and 30,000 samples can be assayed per ml of serum. The sensitivity of this RIA is more than 10,000 times greater than that of any of the present analytical methods (24,25,26,27). Data calculation is done on a programmable computer and results are available within a few minutes.

In the specificity tests, it was observed that only deoxylimonin (27%) and deacetylnomilin (66%) cross reacted with the antibody. However, previous studies (28,29) on the relative concentration of these two compounds in citrus tissues showed that deoxylimonin is present at only 0.47% that of limonin and, therefore, will not be detected in diluted samples. Deacetylnomilin is also one of the minor constituents of the total limonoid fraction and will not contribute to limonin values.

In a study of the distribution of limonin within various grapefruit tissues (Fig. 3), the values obtained were in good agreement with values available in the literature (27,29,30) and the concentration gradient from growing leaves into the fruit conductive tissue and ultimately the seed supports the recent results obtained by Hasegawa and Hoagland (31). Although an extensive quantitative analysis of both fruit and vegetative tissue and the kinetics of accumulation have not yet been done, with the development of an RIA for limonin these studies are now possible.

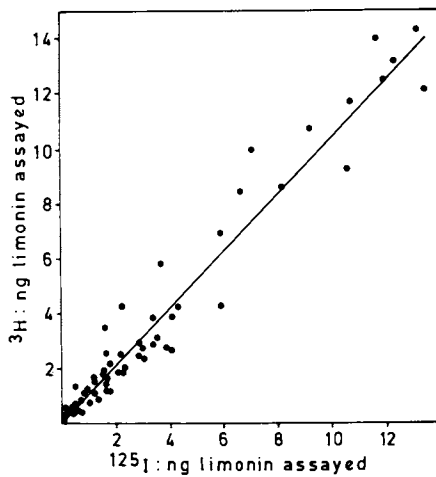
The second RIA system was developed using ^3H -limonin as a tracer. Establishment of a second system was important since many laboratories possess scintillation equipment for counting beta emitting isotopes, but gamma counting equipment is less readily available. In addition, the stability and half-life of a tritiated tracer is very long compared to ^{125}I and, thus, a single radiosynthesis can produce enough tracer for several years or more.

The characteristics of the original assay are: 1) a detection limit of 0.22 ng or 2.2 ppb, 2) the standard curve is linear over a range of 0.5-100 ng (Fig. 2), 3) no purification of the extracts is necessary, 4) coefficients of variation for triplicate determinations (B/Bo) throughout the measuring range are $3.0 \pm 1.8\%$ and 5) recovery is 97%. The titre of the antibody



Phytochemistry

Figure 3. Distribution of limonin in leaves, stems, and fruit parts of grapefruit (*Citrus paradisi*) (22)



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Figure 4. Scatter diagram for various plant extracts assayed by both the ^{125}I and ^3H RIA ($n = 67$; $r = 0.95$; $y = 0.96x + 0.07$) (23)

was 400, which means that 4000 samples could be assayed per ml of serum. Since the final titre of the serum is a function of the specific activity of the tracer used, the titre value could be increased by using higher specific activity tracer. Thus, when a second ^3H -limonin was synthesized (108 Ci/mmol vs 22 Ci/mmol for the first) the titre of the antibody was found to increase to 1,500. In comparison with the ^{125}I assay there also was a high degree of correlation when extracts from various tissues were assayed by both tracers (Fig. 4).

HPLC/RIA Correlation. We compared the limonin content of 180 samples of canned, single-strength grapefruit juice which had been analyzed by HPLC at the Lake Alfred Experiment Station with our ^3H RIA system (Fig. 5). A paired t test showed no difference between the two methods and a linear regression gave $r = 0.794$. We were able to analyze these samples in duplicate in a single working day, whereas the same analyses took more than two months using the HPLC. It was also observed that 78% of the RIA values were higher than the corresponding HPLC, but since the HPLC requires extensive extraction and prepurification prior to analysis whereas the RIA is done with crude extracts, this result is perhaps predictable.

Due to the sensitivity of the RIA, it is now possible to measure limonin concentration in any part of the plant from embryos to small leaf discs, seed coats or any other desirable site or sample. In this type of analysis or screening study it is possible for a single person to analyze 2000 or more samples per week and it is ironic that "sample taking" is now likely to become the limiting step in any large scale study.

In the recent studies of Hasegawa and Hoagland (31) it was found that limonoids (in the form of the A-ring lactone) are synthesized in the leaves of trees and are translocated to the fruit. This observation makes the RIA very applicable in a screening program. From very recent work in our laboratory, we have found that it is actually possible to measure both the A-ring lactone and limonin within a single tissue sample using just the limonin antibody. We have found that the A-ring lactone has a very low cross reactivity with the antibody, therefore if we measure the limonin in neutralized extracts and then acidify these same solutions and again measure the limonin content, the difference will reflect the concentration of A-ring lactone which was present (32). Thus the studies of both compounds can be expanded and the kinetics of each followed as a function of both leaf development and growth. In addition, the effect of environmental and physiological factors can also be analyzed.

ARIA

To the standard RIA described above, another dimension has recently been added with the development of an autoradiographic

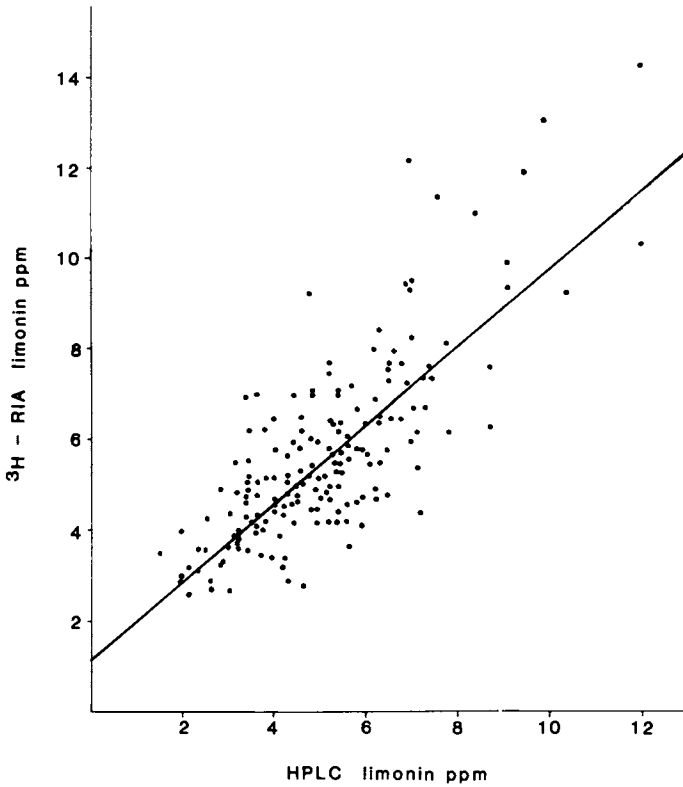


Figure 5. Scatter diagram for canned single-strength grapefruit juice assayed by ³H-RIA and HPLC (n = 180; $r = 0.794$; $y = 0.844x + 1.381$)

immunoassay (ARIA) (33). In this modified RIA, the purpose is not to measure with extreme accuracy the content of substances, but rather to merely identify those plants, cell cultures or juice samples within large populations which contain high or low amounts of the compound under study. In this procedure the RIA principle is employed but, rather than doing radioactive counting on each sample, the amount of radioactivity is determined on X-ray film. By adjusting the dilution of the antibody the procedure can be made selective for amounts of substances below or above a particular level. By using ^{125}I as the radioactive tracer as little as 3,000 CPM can be detected after a 20 hr film exposure. The procedure's real usefulness comes from the fact that whereas the RIA can process 400-800 samples per day the ARIA can easily screen 2,000-3,000 or even more per day. Thus by sacrificing the sensitivity by a factor of about 10, the number of samples which can be measured per day can be increased by the same value. The sensitivity of this assay is in the 1-10 ng range which is still sensitive enough for intact plant, cell culture or juice sample analysis.

Immunoassay for Naringin

At the present time we can only include preliminary results on this assay as its characterization is still in progress. In the determination of bitter flavanone glycosides the basic importance is that the assay is able to distinguish between the bitter disaccharide, neohesperidose, linkage (glucose 1-2 rhamnose) and the non-bitter rutinose linkage (glucose 1-6 rhamnose). From the serum pools which are just being harvested we have been able to ascertain that the antibody being produced is completely specific for the neohesperidose and no cross reactivity has been observed for the rutinose at concentrations up to >5,000 ng/0.1 ml.

Synthesis of an immunogenic naringin molecule was also done using a hapten-protein conjugate and complete details of the antibody production procedures and characterization studies will be published in depth at a later date. However, it is both important and encouraging to report that through use of the immunoassay, major progress in quality control improvement in citrus now becomes a distinct possibility. As a cautionary note, however, it should also be realized that much work, optimization and testing remains to be done before the full potential of these assays can be realized.

Enzyme immunoassay

In the previous pages we have described, in principle, an immunological assay for the quantification of limonin and naringin in citrus samples. In these assays we have employed radioactive tracers (^{125}I and H) and have found that both are useable in a

research laboratory situation. As with any assay method, however, the RIA also has several major limitations especially when trying to employ such a technique in commercial citrus processing operations. First, and most importantly, it is undesirable and potentially unsafe to use radioisotopes in the vicinity of foods; secondly, the personnel using these compounds must be rigorously trained in their use and routine health and contamination surveys must be done. Thirdly, the equipment needed to measure radioactivity is expensive to purchase and maintain. Thus, although the RIA method is undoubtedly the preferred system in an isolated research laboratory situation, an alternative method would be most acceptable in practical, "in house" applications.

As mentioned earlier, the immunoassay can be done with a number of procedural modifications and in this instance one must substitute the isotope with another molecule (fluorescent dye, magnetic particle, enzyme) which can be measured and therefore serve as the source of tracer. For our initial studies we have chosen to use the enzyme immunoassay (EIA) system. At the present time the EIA is still in its infancy and although a number of successful EIA's have been developed the method cannot be considered a panacea (34). The future of this assay appears to be very bright and exciting, and there is considerable interest in the application of the EIA to problems in both microbiology and clinical medicine (34). Many of the procedures and protocols are derived from RIA procedures and the EIA, like the RIA, has the potential to be performed in a multitude of procedural variations; but, for the purpose of this manuscript we will describe only the system we have chosen for our use.

Consider, for example, a sensitive competitive assay where the antibody is immobilized on the surface of a polystyrene tube or cuvette with labelled and unlabelled antigen competing for the antibody sites. In this instance the labelled antigen is limonin covalently bonded to an enzyme molecule and unlabelled antigen is the limonin present in juice sample. We have done a number of preliminary limonin-enzyme coupling analyses and of the enzymes we have used (β -glucosidase, alkaline phosphatase and horseradish peroxidase) we have chosen the peroxidase (HPR) as our test system. At present our data are still in the preliminary stage and thus it would be somewhat premature to attempt to present many findings at this time. We have, however, been able to demonstrate that the assay does function and moreover we envision being able to develop this system to a point where it could be used in both a research laboratory as well as in quality control monitoring centers.

In Figure 6 the procedures or steps involved in an EIA are diagrammed. For this assay it is first necessary to bind the antibody to the walls of a polystyrene tube. (These tubes could be made available through several commercial laboratories or could be prepared by laboratory technicians.) For the assay, a sample of juice is incubated together with the tracer antigen.

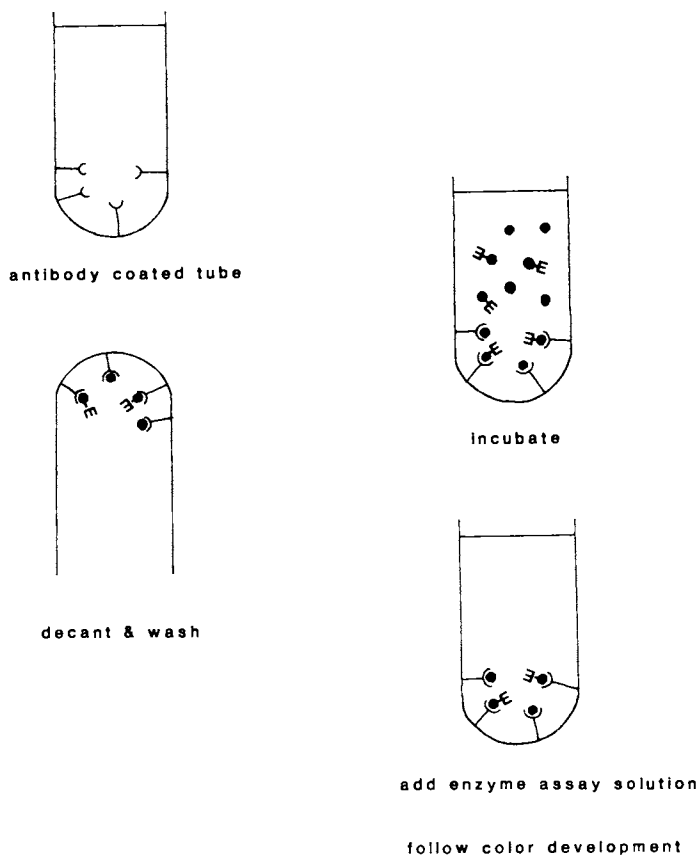


Figure 6. Diagrammatic scheme for performance of a solid-phase enzyme immunoassay ((●) juice sample (limonin); (●-E) tracer (limonin-enzyme conjugate))

The tube is then decanted, washed twice or more with buffer and then an enzyme assay solution is added and the color development or change in absorbance is measured. The amount of enzyme remaining bound to the antibody will be a function of the amount of antigen present in the juice sample and, thus, the color development can be used to calculate the amount of juice antigen. In theory this assay would be quite easy to use, it would be rapid, safe and the only equipment necessary would be a satisfactory spectrophotometer or colorimeter. Work is currently progressing toward a complete characterization and optimization of this assay.

Immunoassay for Orange Juice

While our work on the RIA and EIA for limonin was in progress, a research group in Israel (35) reported on an immunoassay for estimating the orange juice content of commercial soft drinks and reconstituted juice. This is of great importance to the citrus industry since adulterated or improperly reconstituted products greatly affect the quality of the product and thus ultimately reflect upon citrus quality control credibility.

A great deal of research effort has already been expended in this area of product reconstitution and the problem seems to be never ending as new methods of adulteration always seem to be emerging. In this new report the authors report that they were able to elicit antibody production by injection of pure orange juice. The test for determination of orange juice content was that of gel diffusion (35) and the detection limits were statistically valid for concentrations as low as 2.5%. From the data presented in this paper, it is evident that this immunological method is more accurate and sensitive than any method presently available. More importantly the assay is cheap, simple, rapid and can be performed in any ordinarily equipped laboratory. Preservatives, colorants, oils and juices of other fruits do not affect the assay. Interestingly, the preliminary studies of the antigen-antibody interaction suggest that the antigen is not a protein.

Conclusions

It is both encouraging and exciting to observe that the field of immunology has finally been directed toward plants and molecules of plant origin. For all of plant science, the future developments of the RIA, EIA and other immunoassays should aid greatly in solving some of our most troublesome problems. We are of the opinion that the immunoassay has the potential to become a major analytical tool in the citrus industry and in Table IV are listed just some of the areas where this procedure might be employed.

Table IV

Uses of Immunoassay in Quality Related Programs

- A) Basic Research:
- 1) Distribution analyses in vegetative parts
 - 2) Distribution and localization within fruit parts
 - 3) Inter- and intra-plant and fruit variation
 - 4) Cultivar variation analyses
 - 5) Stock/Scion relationships
 - 6) Geographic & nutrient effects on quality
 - 7) Crop improvement studies (selection of individual plants possessing improved quality trait(s))
 - 8) Tissue culture studies (cell line selection)
 - 9) Biochemical studies of compound synthesis and metabolism
 - 10) Early detection of bacterial and viral infections
 - 11) Seasonality studies
- B) Applied Research:
- 1) Use in test houses to monitor incoming fruit
 - 2) Measurement of compound levels in the finished single strength and concentrate products
 - 3) Use in blending (e.g. navel juice with non-bitter orange)
 - 4) Bacterial contamination-detection of organisms or compounds
 - 5) Pulp wash studies - adulterated juice

Since an immunoassay can be developed for each compound or antigen, it is conceivable that within the not too distant future we will have many such assays available for both basic research and applied uses.

Acknowledgement

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Analysis of Trace Metals in Orange Juice

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Orange juice has become over the years one of the most widely accepted natural source beverages. Its wide acclaim is due to its invigorating flavor, its source of quick energy in the form of natural sugars, its other nutrient qualities, especially as a source for vitamin C, and its high potassium content relative to other inorganic metals, particularly sodium. This latter attribute (high potassium low sodium) makes orange juice a highly desired food source for those who must restrict the intake of sodium in their diet. The authors' interest in orange juice is, however, not in the nutritive area but in formulating a complete description of the elemental composition, especially the trace inorganic elemental composition, and how this composition relates to geographical origin.

Orange juice is marketed in the forms of single strength (usually reconstituted from concentrate) and as frozen concentrate. In Florida these forms are defined in degrees Brix (a value which relates to the sugar or total solids content). In most instances, in this paper, concentrations of constituents will be given based on "single strength" juice, a term which denotes the concentration at which the juice is usually consumed regardless of the form in which it was purchased.

There are various expressions relating to elemental composition that describe the magnitude or concentration range of the particular element under consideration. In the literature, one finds expressions "major", "principal", "minor", "trace", "ultra-trace" and others relating to concentration levels. In this discussion we will adopt terminology for three categories; macro (those elements above 1%), major trace (elements from 10 ppm to 1%) and minor trace (elements less than 10 ppm). These range terms are purely arbitrary and are not meant to be offered for adoption as a generally accepted terminology set. There possibly should be one more category such as ultra trace for those elements which may be present but are at concentrations too low to be detected by modern day instrumentation. For the present discussion, however, this range has little value.

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Water, sugars, organic acids and amino acids make up all but a few tenths of 1% of "single strength" orange juice. A breakdown of the composition of orange juice is presented in Table I.

Table I. Components of Single Strength Orange Juice

<u>Principal Constituents</u>		<u>Principal Constituents of Major Elements (Column I) and Other Elements (Column II)</u>			
		<u>I</u>		<u>II</u>	
Water	86%				
Sugars	11.5%	Oxygen	83.85%	Potassium	0.2 %
Amino acids	1%			Nitrogen	0.1 %
Organic acids	1%	Hydrogen	10.65%	Phosphorous	0.02%
Inorganic Ash	0.5%			Magnesium	0.01%
		Carbon	5.15%	Calcium	0.01%
				All Other Elements	0.01%
TOTALS	100%		99.65%		0.35%

The values in Table I are approximate and will depend on geographical locale, soil conditions, fruit variety and fertilization practices. It is the group of elements comprising less than 0.01% of single strength orange juice that will be the principal subject of this discussion.

Historical

The recognition in the early part of the twentieth century that certain mineral elements in small concentrations had observable effects on plant, animal and human well-being has led a host of investigators to try to measure these effects quantitatively. A remarkable and noteworthy beginning leading to the continued modern day interest in applying analytical spectroscopic methods to the analysis of agricultural products was made by Henrik Lundegårdh in the late 1930's and early 1940's. Lundegårdh was the first to establish the utility of flame and spark spectrographic methods for soil and leaf analysis (1,2,3,4). The results of this work indicated a relationship of the soil content of major trace nutrients like calcium, phosphorus and nitrogen and minor trace nutrients like manganese, copper and iron to optimum plant and fruit development. It has now been well established by the Citrus Experiment Station, Lake Alfred, Florida, (5), that these elements along with boron, magnesium, molybdenum, potassium and zinc are essential elements in the promotion of satisfactory growth of orange trees and the production of fruit.

Because of the important nutritive attributes of the eleven elements specifically listed above, most of the analytical effort in the past few decades has been directed toward the determination of the concentrations of some or all of these elements in foods and plants of interest. However, there can also be justifiable interest in what other elements are present. Some elements are, of course, toxic even at low concentrations. Some elements may have as yet undiscovered nutritive value. Beyond these considerations it may be important to know if there are elements whose concentration depends on, and thus may be indicative of, the geographical locale in which the plants were grown. If such elements are present and if their concentrations can be measured, this information, perhaps, could be used for "fingerprinting" or characterizing the juice as to its origin.

The literature is replete with studies on the measurement of a limited number of elements present in soil, plant, leaves and fruit; and estimates have been made of the relationship of this content to growth, maturity and quality of oranges. In the last 13 years (taken from Chemical Abstracts), the authors were able to account for nearly 100 references relating to this subject. Many of these references, however, were from obscure foreign publications and only a few presented meaningful comparisons of juices from different sources. In a fairly recent review, Kefford and Chandler (6) gave a comprehensive account of geographical comparisons which had been made to that date (See Table II).

An examination of these comparisons reveals that they deal mostly with the more common nutrient elements like calcium, phosphorus, potassium and nitrogen. An interesting aspect of this compilation is the wide range in concentration values for elements in juices from different locations throughout the world. Of the geographical areas studied by us, these wide variations have not been observed. A selection of references from the extensive literature is given at the end of this chapter (7-22).

There have been only two comprehensive studies made on the elemental content of orange juices produced in the United States. The first of these was reported by Roberts and Gaddum (23) a little over 40 years ago. That study was a spectrographic analysis of the elemental contents of several varieties of Florida oranges and grapefruit which were grown during that period of time. Excerpts from their results are shown in somewhat modified form in Table III.

It is noteworthy that the results of the Roberts and Gaddum study compare remarkably well for most elements with modern day results obtained by us using the more sophisticated instrumentation (see Table VII). This can be observed by comparing the value for Valencia oranges with the modern values since the Blood, Seedling, and Lue Gim Gong varieties are no longer commercially produced in Florida.

The only other comprehensive study was reported in the early sixties by Birdsall et al. (24). Birdsall and coworkers, like

Table II. Inorganic Constituents of Orange Juice from Various Locales^a

Total Ash	K	Na	Ca	Mg	Fe	P	N	Cl
(mg/kg)								
Orange								
Whole ^b	920-2780					130-280	1200-2460	
Juice ^c	1800-4600	2-30	49-206	50-147	.5-6	42-240	570-1800	20-55

^aFrom Kefford and Chandler (6)

^bExtremes of ranges of fruit from Israel, Spain, South Africa, U.S.A., West Indies, and South America.

^cExtremes of ranges from Israel, California and Arizona.

Table III. Elemental Content of Several Varieties of Florida Orange Juices (PPM)^a
(1937)

Element	Seedling Orange	Blood Orange	Valencia Orange	Lue Gim Gong Orange	Tangerine
aluminum	4	8	6	3	3
barium	0.36 - 1.4	3.8 - 15.0	0.03 - 0.12	0.004-0.040	0.32 - 1.2
bismuth	N.D.	N.D.	N.D.	N.D.	N.D.
boron	0.13 - 0.37	0.23 - 0.48	0.041 - 0.21	0.03 - 0.12	0.12 - 0.30
calcium	160	110	90	110	140
cadmium	N.D.	N.D.	N.D.	N.D.	N.D.
chlorine	30	30	30	30	30
chromium	0.03 - 0.14	0.04 - 0.15	0.03 - 0.13	0.004-0.040	0.03 - 0.12
cobalt	N.D.	N.D.	N.D.	N.D.	N.D.
copper	0.36 - 1.4	0.38 - 1.4	0.33 - 1.3	0.19 - 0.39	3.2 - 12.1
iron	2	5	1	1	2.8
lead	N.D.	0.03 - 0.14	N.D.	TRACE	TRACE
magnesium	100	60	50	50	70
manganese	0.36 - 1.4	0.38 - 1.4	0.20 - 0.41	TRACE	1.2 - 3.2
molybdenum	N.D.	N.D.	N.D.	N.D.	N.D.
nickel	N.D.	TRACE	N.D.	TRACE	TRACE
phosphorus	300	280	320	300	150
potassium	1870	1850	1720	1570	1770
silver	N.D.	TRACE	TRACE	TRACE	TRACE
sodium	80	90	70	70	60
sulfur	30	50	40	20	60
strontium	3.6 - 13.6	0.03 - 0.14	0.004 - 0.04	0.004-0.040	0.03 - 0.12
tin	0.03 - 0.14	0.37 - 1.4	TRACE	N.D.	0.03 - 0.12
titanium	N.D.	0.14 - 0.38	0.004 - 0.04	0.30 - 1.2	.004-.04
vanadium	N.D.	N.D.	N.D.	N.D.	N.D.
zinc	0.45 - 2.3	0.14 - 0.38	0.33 - 1.3	0.11 - 0.31	0.12 - 0.32
zirconium	N.D.	0.03 - 0.14	TRACE	TRACE	0.03 - 0.12

^aConcentrations given in parts per million based on single strength juice. See ref. (23).

Roberts and Gaddum, reported on elements measured spectrographically; except their measurements were done on oranges and lemons grown in California. Thirty-one elements, all of which were metals, except boron and phosphorus, were measured. The results were reported in categories as percent of the ash of the juice. Selecting only the juice data, rearranging it slightly, and expressing it in terms of parts per million based on the original juice instead of as a percentage of the ash, the values appear in Table IV.

Table IV. Elemental Content of California Valencia and Navel Orange Juices (PPM) (1961)^a

Juice Type/PPM of Juice Sample	Greater than 40 PPM	Concentrations between 0.4 PPM and 40 PPM	Trace Less than 0.4 PPM	Not Detected
Valencia	Ca, Mg, K	P, Na, Si, Fe B, Sr, Al	Mn, Cu, Ti, V, Cr, Ni, Mo, Sn, Zr, Co, Ba, Zn, Sr	Bi, Cd, Pb, Ag, Sb, As, Cb, W
Navel	Ca, Mg, K	P, Si, Na, Al, Fe	Ti, Cu, Ni, Cr, V, Mn, Zn, Li, Zr, Sr, Ag, B	Bi, Ba, Co, Sb, As, Cb, W, Pb, Mo

^aData from Birdsall et al. (24).

As one can readily see, the quantitative scale of the results of Table IV is less definitive than the scale in Table III. However, order of magnitude agreement is apparent from a comparison of the two sets of data showing that there are probably no gross differences in the metal contents of juices from the two sources - Florida and California. It will be noted that the concentration range for phosphorus is an order of magnitude less in this data than the Roberts and Gaddum data and, judged by that and the values we obtain, we conclude the range for phosphorus in Table IV to be in error. It could be stated here that Lundegårdh did most of his studies on soil and leaf analysis contending that the fruit (in this case the orange itself) varied little in composition.

In recent publications, we (25,26,27,28) have discussed some modern approaches to the analysis of orange juice, and the results of these studies will be presented in the following sections of this chapter.

Methodology

In any analytical problem the analyst must outline the goals to be achieved by the analysis before deciding on methodology. Often it is desired to measure the concentration of perhaps, one, or at most, just a few elements. In these circumstances, the investigator may, for example, be interested in applying an element like manganese or zinc to the soil or fertilizer and monitoring the resulting influence on orange tree development or fruit quality. Alternatively, the objective behind a particular analysis might be to measure the contamination levels of elements like cadmium and lead which result from the invasion of the growing area by foreign environmental agents.

In situations like these the method of measurement of the element (or elements) of interest can be tailored specifically to suit the particular needs. As a matter of fact, for a few metals like mercury and tin and the metalloids arsenic, selenium, antimony, and tellurium special methods of sample preparation are usually required (29,30,31,32,33). The reason special precautions and special methods are required for the elements listed above, and for certain others, is that they either are readily reduced to volatile forms, react chemically to form volatile compounds, or are influenced in their analysis by the matrices usually present.

Sample Preparation

Orange juice in concentrate form (the form usually selected for analysis) is generally about four times the concentration of single strength juice and therefore is primarily sugar and water (roughly 50/50; see Table I). Usually, for analytical purposes, the organic matter must be destroyed or broken down in such a way that it does not interfere with the analysis. Considerable emphasis has been given to this aspect in the literature on food and biological analysis. No clear consensus of opinion seems to prevail on what the best approaches are. Probably the simplest method is to dry a suitable sample under a lamp or in an open draft oven until the water is driven off and then ash at temperatures up to 550°C. Although the total time involved in this ashing procedure is usually the better part of one day, this method requires little operator attention and a number of samples can be carried through the process at one time. An additional advantage to ashing is that relatively large samples can be used (up to 20g in most instances). Disadvantages are that volatile elements (or elements forming volatile compounds) can be lost through volatilization. Depending on what other ions are present, the list of elements generally lost by ashing certainly will include elements like mercury, arsenic, cadmium, selenium, antimony and tellurium. Some losses, also depending on the circumstances, may be observed with metals like silver, lead and possibly also copper and zinc

although the authors have not observed significant problems by dry ashing when dealing with the small concentrations of silver, copper and zinc present in orange juice. This is probably due to the retentive effect of phosphate since phosphorus is present in orange juice in relatively massive amounts compared to the concentrations of the latter three elements. Also, the high alkali metal content of orange juice, especially the high potassium concentration renders the final ash alkaline, principally as the carbonate. This matrix is also conducive to retention of metals which might otherwise be volatile or be substantive on the ashing crucible. Silica crucibles are less desirable for ashing than platinum ones. This results from two considerations: 1) the silica surface has open reactive sites for ion exchange and/or ion absorption and 2) silica tends to pit and erode into the highly alkaline matrix of the final ash.

A low temperature ashing technique has recently begun to receive attention (34,35,36,37,38). The samples are placed under vacuum (after removing the moisture) in an atmosphere of oxygen or a mixture of oxygen and freon. The latter gas seems to speed up the ashing process. Heating is provided by high frequency coils surrounding the ashing chamber. The chamber and ashing containers must be made of quartz if freon is to be used as a component of the gas atmosphere. The authors have verified that up to 10 g of frozen concentrated orange juice in multiple units may be ashed in a suitably sized chamber. The temperature of the ashing process can be varied up to 200°C depending on the frequency power. It is claimed that many of the metals considered to be volatile at higher temperatures are completely or nearly completely retained by this ashing method (38).

A number of discussions (32,39,40,41,42) can be found in the literature relating to the merits of dry versus wet ashing. Wet ashing entails decomposing the organic components of the sample by means of mixtures of oxidizing acids. The usual mixtures are nitric and sulfuric acids; nitric and perchloric acids; nitric, sulfuric and perchloric acids; and sulfuric acid and hydrogen peroxide. There are two principal advantages to acid ashing (so called wet ashing): 1) there are little if any losses by volatilization; and 2) the elements are kept in an oxidized state and in a strongly acid medium; thus, diminishing, if not eliminating, the chance of substantive reactions or reactions with the surface of the container. On the other hand, there are some very serious considerations which tend to rule against wet ashing. The volume of acid to weight of sample is high, usually on the order of 15-10 ml (or more) of acid per gram of sample especially when dealing with orange juice. This means that if one wishes to decompose say 10-20 g of sample for a survey of many elements, the volume of acid needed is large, and because of the hazardous nature of mixtures containing perchloric acid, considerable time and constant attention is demanded of the operator. Also, with orange juice in the presence of strong acids, considerable foaming

occurs at the beginning stages of the oxidation, and it becomes necessary to allow the samples to react slowly at low temperatures until the foaming tendency is subdued, a period of time that may take several hours. The copious amounts of acid needed increase the likelihood of metal contamination from the acids themselves since it is nearly impossible to remove all traces of impurities during their manufacture. Furthermore, one runs the risk of the acid anions reacting with the metals to form relatively stable but insoluble products, e.g., barium, calcium and other alkaline earths form sulfates of varying degrees of insolubility. If one or just a few elements are to be measured and if it is determined that volatile losses occur such as with mercury, arsenic, antimony and group VI elements, then acid ashing offers an acceptable way for preparing the sample. If a broad range of elements are to be measured, and interest in the above list of volatile ones is minor then we consider dry ashing to be the method of choice, especially for preparing orange juice for analysis. These considerations bear out the thought expressed earlier that methodology must be tailored to the specific analytical problem.

For a selected list of elements (26) there is another acid treatment procedure which is readily applicable to the analysis of orange juice; this procedure involves hydrolysis with moderately strong nitric acid to breakdown most of the sugars and to decrease the size of the pulpy constituents. The solution is then filtered, diluted, and measured by atomic absorption. For elements that can be determined with an air-acetylene flame using a high solids (three slot) burner, this procedure offers a useful alternative. Fricke et al. (33) also mentions the utility of this method and gives comparative results on the use of this method in sample preparation.

If dry ashing has been used, or if the samples following acid ashing have been evaporated to dryness, the final steps in the preparation of the sample for analysis will depend on the type of end measurement to be used; these end measurements will be dealt with in some detail in the discussion to follow.

End Measurement Techniques

The final measurement step can be done in a number of ways. A few of the elements in orange juice are present in large enough concentrations and/or have chemical characteristics which permit the use of classical analysis. Phosphate and some heavy metals like zinc and iron may be measured colorimetrically. Calcium has been measured as the oxalate, but such methods are time consuming and may require prior separation from the inevitable matrix before analysis.

Atomic Absorption Spectrometry. The most widely accepted measurement techniques in modern day analysis are found in the field of spectroscopy. A search of the literature reveals that

atomic absorption spectroscopy is the most popular modern technique for the measurement of trace metals in foods (26,30,32,33,41,42,43,44,45,46). Crosby (43) in a comprehensive review article surveyed the literature over a five-year period using two appropriate journals (one British and one American) and found that there was a total of 83 articles dealing with atomic absorption as compared to a combined number of 62 dealing with molecular absorption spectrometry, flame photometry, neutron activation, x-ray fluorescence, chemical titration, etc. Atomic absorption certainly has advantages because of the extensive and authoritative studies that have been made to improve instrumentation and work out the details for the analysis of metals in many types of chemical matrices. Methods have been worked out for some 60 to 70 elements covering certainly the most important ones in the periodic table. Modern atomic absorption instrumentation has been developed which almost completely eliminates the possibility of operator error, and with multi-element lamps available, a degree of multielement capability has been incorporated. This minimizes the drudgery of replacing lamps and reoptimizing instrumental conditions for each new metal to be analyzed. Atomic absorption can also be made very sensitive by the use of the graphite furnace instead of a flame as a sample cell (30,47,48). Furnaces have been used in a number of different forms and configurations ranging from cells with open tops to filaments and tube designs. Samples can be in the form of solids, pastes, or liquids, and by programming the heating of the furnace, preliminary ashing can usually be avoided.

Atomic Emission Spectrometry. Emission spectroscopy was the earliest developed multielement measurement technique (49,50,51,52). Its widest acceptance was by the metal industry where it was particularly useful in determining a few elements repetitively in a metal (usually some form of steel) matrix which was well defined. It was also used in the food and agricultural field and was responsible for much of the early knowledge of the concentrations of a number of trace elements in orange juice (23,24).

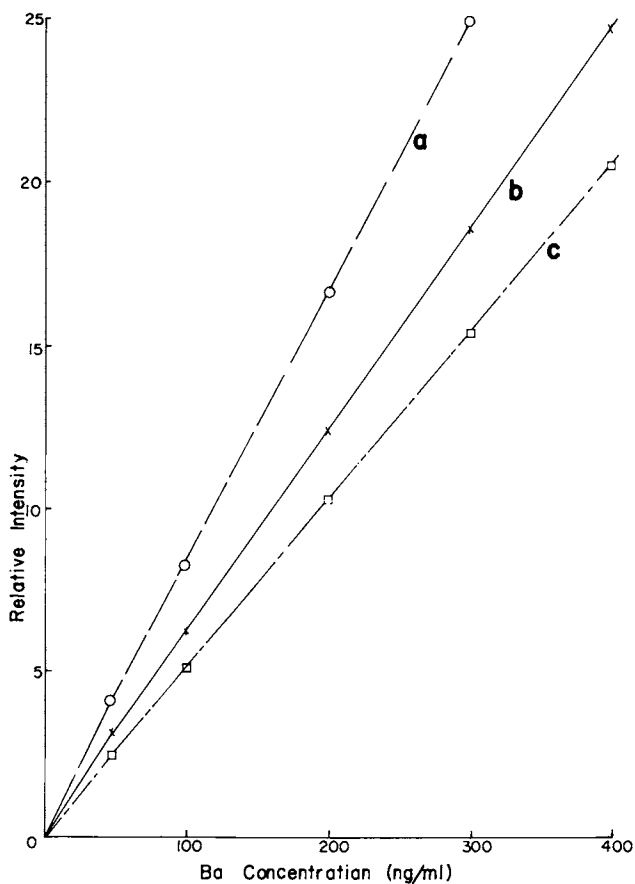
Recently, a very important development has been made to enhance the ease of manipulation and the range of applicability of emission spectroscopy to trace metal analysis. This development is the plasma source which can be employed as an accessory source in most direct reading emission spectrometers in place of the arc or spark or may be incorporated directly in the design of the spectrometer by the manufacturer. This development has been discussed in detail in the recent literature (49,53-58).

There are two popular types of plasma sources: 1) the direct current plasma (DCP), and 2) the inductively coupled plasma (ICP). In the commercial version of the former plasma source (marketed by Spectrometries, Inc.), the sample is aspirated with argon through a small orifice into a chamber where the large droplets settle out and the fine mist is conveyed by the argon stream through a chimney to the vertex of a plasma which is in the form of

an inverted Y configuration. The vapors are heated and atomic emission takes place in this area (vertex of the Y). This emission flux is focused on an entrance slit to the monochromator and progresses through the optical path which consists of a system of mirrors, an echelle grating and a prism to separate the wavelengths into a two-dimensional pattern consisting of several wavelength orders. Measurement of intensities can be simultaneous (i.e., multielement) or sequential depending how the instrument has been designed to operate.

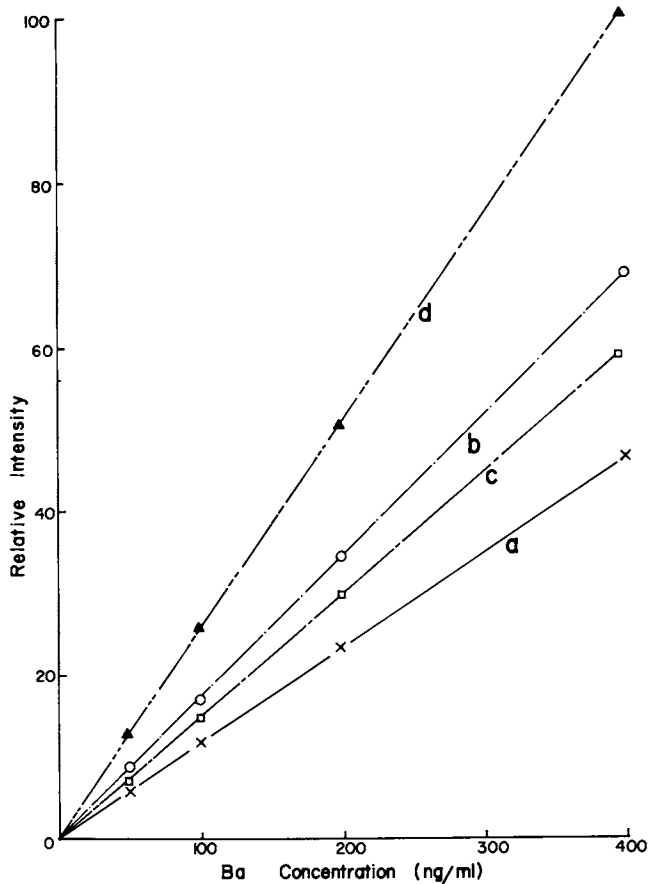
The other type of plasma source, the inductively coupled plasma, has been engineered into specifically designed instrumentation modules by Applied Research Laboratories, Jarrell Ash (a division of Fisher Scientific Corp.), Perkin Elmer, Instrumentation Laboratories, and Baird Atomic in the United States; foreign models are also available. When the ICP plasma is in operation, the sample is dispersed as liquid droplets into an argon stream and directed to the center zone of the hot plasma. This operating mode is different from the direct current commercial version and it means that the excitation region is probably considerably higher in temperature. There are some advantages and some disadvantages to the ICP system compared to the DCP system. Elements, like vanadium, molybdenum and tungsten which tend to form highly refractory compounds are probably more completely atomized. Therefore, there is less interference from the matrix, a type of interference which is nearly always a problem with spectroscopic analysis. The principal drawbacks are: 1) nebulizer design is critical in the operation of inductively coupled plasmas; the reason is that close control of the rate of flow of sample to the plasma must be achieved to avoid plasma instability, 2) the viewing (observation) area to which the optics are exposed is directly in the center of the plasma and background emission is high compared to the situation with the direct current plasma where very little of the plasma region is observed during analysis, 3) multielement facilities, the type of instrumentation usually accompanying the inductively coupled plasmas, are expensive and may be out of the financial resources of many small laboratories, and 4) when a multielement facility is chosen the buyer must make a decision as to which elements he is interested in determining; in doing survey work this decision is hard to make because one seldom knows in advance the elements that may be important.

Because of the disadvantages of the ICP systems listed above, we chose to do our study on the simpler less expensive sequential direct current plasma system (27, 59-65). It is well to recognize that in any choice of this kind trade-offs may become necessary. For example, the DC plasma is subject to more or less severe matrix effects, and these must be accounted for in setting up the methodology (28). These effects are illustrated in Figures 1 and 2 which show the influence of the large potassium concentrations on both the atom and ion lines of barium.



Analytical Chemistry

Figure 1. Effect of potassium on barium calibration curves using DCPAES: (a) analytical curve for barium in matrix containing constant K concentration (800 ppm) in 0.1M HNO₃; (b) analytical curve for barium in matrix containing constant K/Ba ratio (4000/L) in 0.1M HNO₃; (c) analytical curve for barium in 0.1M HNO₃ (28)



Analytical Chemistry

Figure 2. Matrix effects on the calibration curve of barium using DCPAES at the atom line (553.55 nm) and the ion line (455.40 nm): (a) barium in 0.1M HNO_3 , atom line; (b) barium in 0.1M HNO_3 containing 100 ppm K, atom line; (c) barium in 0.1M HNO_3 , ion line; (d) barium in 0.1M HNO_3 containing 1000 ppm K, ion line (28)

Atomic Fluorescence Spectrometry. A spectroscopic technique related to some of the types mentioned above is atomic fluorescence spectrometry (AFS). Like atomic absorption spectrometry (AAS), AFS requires a light source separate from that of the heated flame cell. This can be provided, as in AAS, by individual (or multielement lamps), or by a continuum source such as xenon arc or by suitable lasers or combination of lasers and dyes. The laser is still pretty much in its infancy but it is likely that future development will cause the laser, and consequently the many spectroscopic instruments to which it can be adapted to, to become increasingly popular. Complete freedom of wavelength selection still remains a problem. Unlike AAS the light source in AFS is not in direct line with the optical path, and therefore, the radiation emitted is a result of excitation by the lamp or laser source.

Atomic fluorescence spectrometry provides high sensitivities for elements whose analytically useful energy transitions are in the ultra-violet regions of the spectrum. This sensitivity diminishes as one approaches the upper end of the visible region (400-700 nm). We made a comparative study (28) determining several elements in seven brands of Florida orange juice by inductively coupled plasma atomic emission spectrometry (ICPAES), direct current plasma atomic emission spectrometry (DCPAES), flame atomic fluorescence spectrometry (FAFS), and flame atomic absorption spectrometry (FAAS). These comparisons are shown in Table V. The samples for this study were dry ashed and dissolved in either nitric or hydrochloric acids, i.e., these were separate sets dissolved in each acid (the acid concentration during analysis was 0.1 M). It is observed that the nitric and hydrochloric acid media show some differences, but the differences do not seem to be particularly consistent. The precision of replication using the instrument types listed was generally the best with FAAS. In spite of the variability noted between some of the methods, the results seem well within similar ranges of concentrations and the degree of conformity gives confidence to the general validity of the results.

Neutron Activation Spectrometry. Another instrumental technique which has applicability to a wide range of elements is neutron activation analysis. In this method the sample (which could be orange juice without any prior sample treatment) is irradiated with a strong neutron flux. The elements of analytical interest are thus converted to unstable isotopes which decay with characteristic energies and thus measurement of the intensities results in analytical values for the elements of interest. There are some serious drawbacks to this method, however. The matrix can cause severe background effects especially when the sample contains large amounts of an element, like potassium, which is the situation with orange juice. In this event tedious chemical separations must be carried out to achieve adequate selectivity, accuracy

and precision. Facilities for neutron activation are very expensive and, thus, most often the analyst would generally be obliged to send samples to a service laboratory equipped to perform such analysis.

A sample of Florida orange juice has been analyzed by neutron activation (66) and the results are given in Table VI. For some elements, actual concentrations are shown along with the precision of the data, but for most elements only maximal concentrations are listed. This means that values can not be higher than those given but could be much lower. Again, these data are included in this discussion only for comparison with that to be presented in subsequent paragraphs and it should be emphasized that the values listed as maxima are relatively meaningless.

For many of the elements, the data agree well within an order of magnitude with the data gathered by us and with the data of Roberts and Gaddum. There are some obvious discrepancies. The upper limits given for sulfur (4000 ppm), phosphorus (2200 ppm) silicon (4000 ppm), and probably Nb (108 ppm) are far too high. Also, there seems to be no reliable evidence that yttrium, titanium and nickel and zirconium are above 1 ppm in single strength orange juice. Otherwise, the data are credible, and it should be noted that these values were obtained from only one sample and no attempt was made to improve the values by radiochemical separations.

Spark Source Mass Spectrometry. Another method for trace analysis probably should be mentioned and that is spark source mass spectrometry. In this technique, the sample in the form of a solid serves as an electrode and vapors, formed by sparking, are atomized and ionized to metal ions which are separated by a mass spectrometer and measured. The equipment is expensive and good results require the attention of a skilled operator. Even under the best conditions order of magnitude agreement of results is about the best that can be achieved.

Comparison of Florida Orange Juice with Juices from Other Geographic Locales

In an attempt to update the earlier studies on orange juice and to try to arrive at some quantitative values for a broad range of elements in Florida and other source juice samples, we undertook the analytical project described in the following discussion. Orange juice sources of primary interest to us were from Florida and Brazil but some values were obtained on juices from other locales, i.e., Mexico and California are included for comparison.

Experimental

Twenty-four Florida juices, seventy-four Brazilian and some

Table V. Comparative Analytical Data From Four Instrumental Methods (28)

	ICPAES ^{b,d}		DCPAES ^{b,d}		FAFS ^{b,d}		FAAS ^{b,d}
	HCl	HNO ₃	HCl	HNO ₃	HCl	HNO ₃	
Barium	\bar{c} ^a	0.027	0.039	0.047	0.053	0.050	0.035
	%RSD	14.6	10.1	4.77	3.52	14.3	12.8
	LOD	0.018	0.013	0.003	0.007	0.010	0.011
	%AD	1.69	0.833	1.26	1.27	1.12	0.591
Calcium	\bar{c}	101.	88.	87.	79.	100.	95.
	%RSD	1.22	2.58	9.33	6.15	3.58	5.73
	LOD	0.060	0.050	0.166	0.147	0.050	0.024
	%AD	0.404	0.444	0.738	0.620	0.474	0.267
Copper	\bar{c}	c	c	0.380	0.332	0.287	0.303
	%RSD			8.53	8.57	5.91	6.83
	LOD			0.025	0.027	0.026	0.028
	%AD			0.985	1.03	0.567	0.256
Iron	\bar{c}	1.860	1.30	1.05	0.815	1.06	0.83
	%RSD	9.20	4.50	16.1	3.40	14.7	11.7
	LOD	0.207	0.063	0.081	0.068	0.165	0.184
	%AD	0.649	0.446	1.24	3.36	2.27	1.68
Magnesium	\bar{c}	142.	119.	97.	103.	121.	115.
	%RSD	5.25	5.88	4.07	1.85	4.60	5.60
	LOD	0.195	0.080	0.151	0.196	0.219	0.187
	%AD	0.592	0.526	0.514	0.603	1.02	1.02
Manganese	\bar{c}	0.264	0.302	0.161	0.169	0.179	0.180
	%RSD	3.31	6.53	5.89	6.92	7.54	8.61

Table V. Comparative Analytical Data From Four Instrumental Methods (Continued)

	ICPAES ^{b, d}		DCPAES ^{b, d}		FAFS ^{b, d}		FAAS ^{b, d}	
	HCl	HNO ₃	HCl	HNO ₃	HCl	HNO ₃	HCl	HNO ₃
Manganese (Contd')	LOD	0.012	0.007	0.008	0.028	0.017	0.002	0.002
	%AD	0.203	0.479	1.59	1.28	1.45	2.01	1.76
Potassium	\bar{C}	2109.	1652.	2288.	1854.	1818.	1859.	1925.
	%RSD	1.54	1.93	4.99	4.94	2.59	4.57	2.43
	LOD	3.39	2.93	1.95	1.03	2.76	2.02	8.36
	%AD	2.16	0.96	1.06	0.21	1.52	2.19	3.78
Rubidium	\bar{C}	0.676	0.376	0.442	0.376			
	%RSD	8.13	9.80	4.52	8.84			
	LOD	0.380	0.128	0.043	0.52	c	c	c
	%AD	0.633	0.542	2.596	2.11			
Sodium	\bar{C}	11.0	9.64	8.31	5.76	7.03	6.59	5.25
	%RSD	12.4	10.1	4.57	5.55	3.27	3.94	2.22
	LOD	0.037	0.017	0.008	0.005	0.006	0.016	0.018
	%AD	1.04	0.72	0.516	1.00	0.987	1.40	1.05
Zinc	\bar{C}			0.353	0.304	0.294	0.281	0.37
	%RSD			2.94	8.25	15.6	6.85	2.72
	LOD	c	c	0.064	0.076	0.045	0.134	0.0009
%AD			6.36	5.14	1.13	0.089	1.41	

^a \bar{C} = Average concentration, ppm; %RSD = % Relative Standard Deviation; LOD = Limit of Detection, ppm;
^b%AD = Average Deviation of Measurement. All concentrations are calculated on the basis of single
strength orange juice (SSOJ).
^cValues not measured, see text. ICPAES = Inductively Coupled Plasma
Emission Spectrometry; DCPAES = Direct Current Plasma Emission Spectrometry; FAFS = Flame Atomic Fluor-
escence Spectrometry; FAAS = Flame Atomic Absorption Spectrometry.

Analytical Chemistry

Table VI. Elements Determined in Florida Orange Juice By Neutron Activation^{a,b}

Elem.	Conc. ppm	Precision (+ or -)	Elem.	Conc. ppm	Precision (+ or -)	Elem.	Conc. ppm	Precision (+ or -)	Elem.	Conc. ppm	Precision (+ or -)
Ag	<0.024		F	<7.7		Na	7.9	1.6	Sm	<0.009	
Al	18.	4.0	Fe	8.6	2.5	Nb	<108.		Sn	<4.5	
As	<0.10		Ga	<0.28		Nd	<0.25		Sr	0.84	0.21
Au	0.0004	0.0001	Gd	<1.93		Ni	<7.8		Ta	<0.025	
Ba	<0.30		Ge	<0.73		Os	<0.03		Tb	<0.005	
Br	<0.33		Hf	<0.0078		P	<2200.		Te	<0.65	
Ca	<160.		Hg	<0.04		Pd	<0.18		Th	<0.013	
Cd	<0.85		Ho	<0.05		Pr	<1.9		Ti	<18.	
Ce	<0.15		I	<0.02		Pt	<0.6		Tm	<0.013	
Cl	24.	5.0	In	<0.035		Rb	0.83	0.19	U	<0.03	
Co	<0.019		Ir	<0.0002		Re	<0.06		V	<0.18	
Cr	<0.19		K	1383.	278	Rh	<6.3		W	<0.3	
Cs	0.009	0.002	La	0.01	.003	S	<4000.		Y	<35.	
Cu	<0.68		Lu	<0.003		Sb	<0.015		Yb	<0.008	
Dy	<0.028		Mg	<85.		Sc	<0.001		Zn	0.73	0.16
Er	<0.33		Mn	0.20	0.04	Se	<0.073		Zr	<43.	
Eu	<0.001		Mo	<0.40		Si	<4000.				

a) Ref. (66)

b) Concentrations are given in parts per million based on single strength orange juice.

six to ten samples each from Mexico and California were included in the study. All the samples were prepared for analysis by dry ashing. Twenty gram samples of frozen orange juice concentrate were dried to carbonization under heat lamps and ashed in a muffle furnace at 550°C until the ashed residue was completely free of any visible unoxidized carbon. The ashing time usually was about 16 hr. This procedure was, in many respects, similar to that recommended by Horwitz for the preparation of agricultural products for spectrographic analysis (67). The final ash was dissolved in 0.1 M HNO₃ (50 ml) and analyzed by plasma spectroscopy. The spectrometer employed was the Spectrametrics, Inc. Spectraspan II equipped with the Spectrajet III plasma source. Generally, for each individual measurement, two readings (15 sec integration time) were averaged and compared to a standard containing the element being determined in a suitable synthetic matrix. This matrix was formulated to correspond as closely as possible to the element ratios and concentrations usually found in orange juice. A recent publication by us (28) discussed the matrix and matrix effects on the analysis.

Results and Discussion

In Table VII the concentration ranges of the elements in the juices from the various sources are listed. In all instances, the concentrations are adjusted by a factor of four to reflect the concentrations as they would appear in single strength orange juice. This factor, however, is not universally accurate since many of the foreign (to the United States) juices contain high solids. In spite of this variability in solids content, for most elements there is considerable overlap in the concentration ranges. There are some elements, though, that do seem to show significant differences in concentration. For example, Brazilian juices have substantially higher barium and rubidium contents than do Florida juices. There are also differences in the manganese and gallium values and in many instances differences in the boron values. In order to avoid the problem caused by differences in solids contents, when comparing these values, we ratio the elemental values of particular interest to the value for zinc in each sample. It will be noted that the values for zinc in all samples seem to be particularly uniform and thus zinc serves as a convenient reference element. This method of ratioing also requires a reference sample (this can be either an actual sample, or one made up synthetically with known elemental concentrations). The way the derivation of the ratio was arrived at and the elimination of the solids factor by use of the ratio is illustrated in the Appendix. The ratio used is:

$$\frac{I_{AY}}{I_{AR}} \cdot \frac{I_{BY}}{I_{BR}}$$

Table VII. Ranges of Element Contents in Orange Juice Samples From Florida, Brazil, Mexico and Calif. (Concentration Values Based on Single Strength Juice)

Element	in ppm	
	Florida Conc. Brazil Conc.	Mexican Conc. California Conc.
Aluminum	0.041 - 0.155	0.206 - 0.736
Arsenic	<0.015	NM ^b
Barium	0.018 - 0.100	0.051 - 0.550
Beryllium	<0.001	NM
Boron	0.629 - 1.79	1.03 - 1.53
Cadmium	<0.01	NM
Calcium	67 - 123	87 - 135
Chromium	0.003 - 0.021	0.004 - 0.011
Cobalt	<0.01	<0.01
Copper	0.239 - 0.460	0.143 - 0.356
Gallium	0.016 - 0.042	0.018 - 0.231
Indium	<0.01	<0.01
Iron	0.641 - 5.58	1.80 - 6.32
Lanthanum	<0.02	0.016 - 0.065
Lead	<0.1	NM
Lithium	0.002 - 0.008	0.003 - 0.005
Magnesium	95 - 140	82 - 149
Manganese	0.173 - 0.316	0.207 - 0.466
Molybdenum	<0.01	0.002 - 0.038
Nickel	0.008 - 0.067	0.009 - 0.223
Phosphorus	124 - 240	104 - 303
Potassium	1520 - 2660	1245 - 2095
Rubidium	0.236 - 0.741	0.630 - 3.403
Scandium	<0.01	<0.01
Silver	0.002 - 0.028	0.010 - 0.132
Sodium	3 - 9	2.93 - 43.3
Strontium	0.095 - 0.979	0.325 - 0.713
		0.098 - 0.364
		NM
		0.099 - 0.327
		NM
		1.02 - 5.10
		NM
		102 - 150
		0.009 - 0.031
		<0.01
		0.321 - 0.421
		0.023 - 0.127
		<0.01
		1.07 - 8.48
		<0.02
		NM
		0.003 - 0.026
		106 - 155
		0.278 - 0.921
		<0.01
		0.016 - 0.062
		193 - 309
		1741 - 2465
		0.757 - 3.73
		<0.01
		<0.01
		2.51 - 9.78
		0.395 - 0.733

Table VII. Ranges of Element Contents in Orange Juice Samples From Florida, Brazil, Mexico and Calif. (Continued)

Element	Florida Conc. in ppm	Brazil Conc. in ppm	Mexican Conc. in ppm	California Conc. in ppm
Tin	0.001 - 0.422	<0.001 - 2.58	0.063 - 2.73	0.016 - 0.884
Titanium	0.002 - 0.022	0.004 - 0.357	0.005 - 0.020	0.012 - 0.025
Vanadium	ND ^a	ND	ND	0.001 - 0.010
Yttrium	ND	ND	ND	ND
Zinc	0.242 - 0.480	0.255 - 0.527	0.267 - 0.449	0.249 - 0.538

^aND = Not Detectable^bNM = Not Measured

where A is the analyte element, B is the reference element (in this case zinc), I is the intensity reading for the respective element, Y is the sample being analyzed and R is the reference sample. The ratios of some elements thought to have significant fingerprint character are shown in Table VIII.

Table VIII. Ratios^a of Selected Elements and Deviations Shown by Brazil Samples Compared to Florida Samples as a Standard Reference Population

Element	Range	Florida	
		\bar{x}	SD
Ba	0.99 - 4.70	2.36	0.722
B	2.07 - 4.70	3.56	0.499
Ga	1.81 - 3.46	2.34	0.268
Mn	2.03 - 3.57	2.85	0.343
Rb	1.51 - 4.05	2.48	0.473

Element	Range	Brazil	
		dev(α)	P ^b
Ba	9.33 - 33.78	9.68 - 43.64	$1.07 \times 10^{-2} - 5.0 \times 10^{-4}$
B	3.04 - 6.48	< 1 - 5.69	$1.00 - 2.82 \times 10^{-2}$
Ga	2.47 - 6.19	0.48 - 14.26	$1.00 - 4.90 \times 10^{-3}$
Mn	3.28 - 8.28	1.26 - 15.97	$6.28 \times 10^{-1} - 3.90 \times 10^{-3}$
Rb	16.12 - 33.87	29.02 - 66.79	$1.20 \times 10^{-3} - 2.00 \times 10^{-4}$

$$^a \left(\frac{I_{AY}}{I_{ZnY}} \div \frac{I_{AR}}{I_{ZnR}} \right)$$

$$^b \text{Tschebycheff's Inequality: } P(|x - \bar{x}| > \alpha s) < \left(\frac{1}{\alpha^2} \right)$$

Treatment of the Data

One prime objective of this study was to be able to analyze a sample of unknown source, and by use of the ratios arrive at a probability that the sample was or was not from Florida. The Florida group of samples was selected as the standard population of reference.

Tschebycheff's Inequality. An average \bar{x} for each of the "fingerprint" elements was calculated for the Florida group and is listed in Table VIII along with the corresponding standard deviation (s). For an unknown sample a deviation α is calculated from its ratio and, using Tschebycheff's inequality (68,69), a probability P can be calculated where $P(|x - \bar{x}| > \alpha s) < \frac{1}{\alpha^2}$. In this equation, P is the probability that the sample tested is from Florida, x is the sample value, \bar{x} is the standard population average, s is the standard deviation of the values of the refer-

ence population, and α is the number of standard deviations represented by $|x - \bar{x}|$ for the unknown sample. Since it can be assumed that the occurrence of any element is an independent event (not related to the presence of any of the others) one could calculate a combined probability $P_{El_X} \times P_{El_Y} \times P_{El_Z} \dots$ where $El_X, El_Y,$ and El_Z are the fingerprint elements. This would give a total probability that the sample in question is a member of the reference population.

To clarify the use of probabilities let us consider the following treatment of illustrative data. A sample that has low concentrations of fingerprint elements has ratios of these elements to zinc that are at the high end of the probability scale. Another sample that has high concentrations of the same elements has ratios that are at the low end of the probability scale as shown by the following two randomly selected Brazil samples:

Table IX. Single Strength Concentration in PPM

Sample #	Ba	B	Ga	Mn	Rb	Zn
1	0.213	0.866	0.029	0.322	3.09	0.249
2	0.622	1.67	0.054	0.555	4.87	0.361

Ratios (Element Concentration/Zinc Concentration)

1	15.63	3.99	3.95	5.91	23.07
2	31.60	5.32	4.66	7.05	31.79

The \bar{x} and s from the standard Florida population are used to determine individual sample probabilities for each of the fingerprint elements. For barium:

Table X. Calculating Tschebycheff's Probability (for Barium)

Sample #	Ratio(x)	$x - \bar{x}$	$\frac{x - \bar{x}}{s} = \alpha$	α^2	$\frac{1}{\alpha^2} = P$
1	15.63	13.07	18.10	327.7	.0031
2	31.60	29.04	40.22	1617.8	.0006

$$\text{Fla } \bar{x}_{\text{Ba}} = 2.56$$

$$\text{Fla } s_{\text{Ba}} = 0.722$$

Sample probabilities are similarly determined for each of the other fingerprint elements. The total probability that any particular sample is a Florida sample is expressed by multiplying the individual probabilities of each fingerprint element:

Table XI. Total Probabilities

Sample #	P_{Ba}	P_B	P_{Ga}	P_{Mn}	P_{Rb}	$(P_{Ba})(P_B)(P_{Ga})(P_{Mn})(P_{Rb})$
1	.0031	1	.0277	.0126	.0005	5.41×10^{-10}
2	.0006	.0803	.0133	.0067	.0003	1.29×10^{-12}

It is certainly evident that barium and rubidium are the most discriminating "fingerprint" elements but in some instances the other elements have been found to contribute in a useful way to the total probability. This total probability represents the likelihood that the particular sample comes from Florida. Notice that in both examples the probabilities are extremely low.

Pattern Recognition. An alternative treatment of the data is possible and has been discussed by some of us (70). This approach involves the application of pattern recognition, a subject which has received considerable attention in the recent literature. Essentially, the technique involves the transformation of the concentrations of the five target (fingerprint) elements into points in 5-dimensional space which is represented by "pattern vector", for example;

$$X = (\{Ba\}, \{B\}, \{Ga\}, \{Mn\}, \{Rb\}).$$

The assumption we make is that similar samples will define points in 5-dimensional space that lie close to each other. The problem is then reduced to one of discriminating between geometric regions or "categories" in this multidimensional coordinate system. This is achieved by the introduction of a suitable decision vector:

$$D = (a, b, c, d, e).$$

Upon obtaining the dot product of these two vectors:

$$D \cdot X = (\{Ba\}a + \{B\}b + \{Ga\}c + \{Mn\}d + \{Rb\}e),$$

we now have a means for deciding within which category a sample belongs.

Using the results in Table XII the proposed pattern recognition method was applied in discriminating Florida from Brazil juice.

Table XII^a (70)

Element	Florida (ppm)	Brazil (ppm)
Ba	0.025 - 0.07 $\bar{x} = 0.048$	0.183 - 0.53 $\bar{x} = 0.36$
B	0.95 - 1.20 $\bar{x} = 1.08$	0.675 - 1.61 $\bar{x} = 1.14$
Ga	0.03 - 0.04 $\bar{x} = 0.035$	0.047 - 0.11 $\bar{x} = 0.079$
Mn	0.25 - 0.315 $\bar{x} = 0.28$	0.30 - 0.60 $\bar{x} = 0.45$
Rb	0.365 - 0.740 $\bar{x} = 0.55$	2.64 - 4.86 $\bar{x} = 3.75$

^aAll values reported are adjusted for variations in solid content and express concentrations to be found in single strength orange juice.

The decision vector chosen was empirically derived and the optimized value was found to be the following:

$$D = (1, -1, -1, -1, 1)$$

The decision vector was formulated such that when the dot product of it and the sample vector was taken, one of two results would be obtained. Either,

1. $D \cdot X < 0$. Therefore, X would be a Florida juice.
- or 2. $D \cdot X > 0$. Therefore, X would be a Brazil juice.

In general, as noted in the previous discussion on probabilities, trace elemental concentrations in juices of Florida origin were found to be lower than those of non-domestic origin. Therefore, three cases were utilized to test the efficiency of the pattern recognition decision vector.

Case I:

Here we wish to distinguish between a Florida juice with the lowest possible values and a Brazil juice with the highest possible values.

$$a) X_1 = (0.025, 0.95, 0.03, 0.25, 0.37) \quad (\text{Florida})$$

$$D \cdot X_1 = (0.025 - 0.95 - 0.03 - 0.25 + 0.37) = s$$

$$s = -0.84$$

$s < 0$ Therefore, X_1 is a Florida juice

$$b) X_2 = (0.53, 1.61, 0.11, 0.60, 4.86) \quad (\text{Brazil})$$

$$D \cdot X_2 = 3.07$$

$s > 0$ Therefore, X_2 is a Brazil juice

Case II:

Here we wish to distinguish between a Florida juice with average values and a Brazil juice with average values.

$$a) X_1 = (0.048, 1.08, 0.035, 0.28, 0.55) \quad (\text{Florida})$$

$$D \cdot X_1 = -0.80$$

$s < 0$ Therefore, X_1 is a Florida juice

$$b) X_2 = (0.36, 1.14, 0.079, 0.45, 3.75) \quad (\text{Brazil})$$

$$D \cdot X_2 = 2.44$$

$s > 0$ Therefore X_2 is a Brazil juice

Case III:

Here we wish to distinguish between a Florida juice with the highest possible values and a Brazil juice with the lowest values.

$$a) X_1 = (0.07, 1.20, 0.04, 0.315, 0.740) \quad (\text{Florida})$$

$$D \cdot X_1 = -0.745$$

$s < 0$ Therefore, X_1 is a Florida juice

$$b) X_2 = (0.183, 0.675, 0.047, 0.30, 2.64) \quad (\text{Brazil})$$

$$D \cdot X_2 = 1.80$$

$s > 0$ Therefore, X_2 is a Brazil juice

Case III is the most important case because it represents the case where maximal overlap between the two origins may exist.

The conclusion of these calculations is that for the worst possible case in which all elements of interest in a Florida sample have their highest possible values and those of Brazil have their lowest, the proposed technique offers a clear and substantial separation of the two groups. With the samples of the population that has been observed to date, the decision vector is 100% successful. These preliminary studies have shown this approach to be a valid one and will undoubtedly be of increasing interest and value in the future.

Summary

We have attempted to assemble from the voluminous literature on trace analysis those approaches which appear to have the most applicability to the study of trace metals in orange juice. Some comparative data has been given and it can be observed that as instrumentation becomes better adapted to such applications better and more useful metal concentration relationships will be discovered.

Appendix

- (1) I_{AY} = Analyte signal for element A in sample Y
- (2) I_{AR} = Analyte signal for element A in reference R
- (3) K_A = Factor to convert intensity of signal for element A to weight of A
- (4) W_Y = Weight of sample Y
- (5) K_Y = Factor to convert weight of sample Y to weight of solids in sample Y
- (6) $\frac{I_{AY} \cdot K_A}{W_Y \cdot K_Y}$ = Weight of element A in sample Y solids
- (7) $\frac{I_{BX} \cdot K_B}{W_Y \cdot K_Y}$ = Weight of element B in sample Y solids
- (8) $\frac{I_{AR} \cdot K_A}{W_R \cdot K_R}$ = Weight of element A in reference solids
- (9) $\frac{I_{BR} \cdot K_B}{W_R \cdot K_R}$ = Weight of element B in reference solids

$$\frac{(6)}{(8)} \div \frac{(7)}{(9)} = \frac{I_{AY}}{I_{AR}} \div \frac{I_{BY}}{I_{BR}}$$

Abstract

Orange juice is a widely accepted nutritive component of the diet of well nourished people in many areas of the world. In spite of this, relatively little is known about the trace mineral content of orange juice and how this mineral content varies with geographical source. Methods for determining mineral elements are presented in this discussion along with some comparative data showing the similarities and differences which occur especially between orange juices from Florida and Brazil. Some statistical representations are given to show how these differences may be used as fingerprints of geographical origin of the juices.

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Methods for the Detection of Adulteration in Processed Citrus Products

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Adulteration of processed citrus products, such as juices and oils, continues to be a serious world-wide economic and regulatory problem. Adulterations of citrus juices have progressed from simple dilutions with water, sugar, and acids to sophisticated methods utilizing adulterants designed to obscure adulteration. In response, regulatory and industrial related agencies concerned with preserving quality have improved or developed methodology to detect adulteration. However, as methodology for detection becomes more sophisticated so does methodology for disguising adulteration.

Numerous surveys in the literature report extensive adulteration of citrus juices in commercial channels. For example, Royo Iranzo and Aranda (1) and Royo Iranzo *et al.* (2) found from one half to one third of the commercial European bottled orange juices tested were adulterated. Mears and Shenton (3) reported the most common form of adulteration in the early 1970's was the use of fruit by-products such as extracts of peel and pulp (pulpwash). It appears that the problem has continued into the 1980's. Three orange juice surveys in the United States (162 samples obtained from retail outlets) conducted by the Florida Department of Citrus during 1979 and 1980 indicated gross adulteration by orange pulpwash (PW) and/or sugars and/or dilution (Petrus *et al.*, unpublished data).

The Florida Department of Citrus rules (4) prohibit the addition of any PW to frozen concentrated orange juice (FCOJ). The U. S. regulations, while not as strict, prohibit addition of PW beyond that obtained from the particular batch of fruit from which the juice was obtained (5). Since Florida produces most of the FCOJ in the United States, this type of adulteration is a national as well as a state problem.

The literature contains considerable citrus compositional data along with many proposed methods of detecting adulterations. However, there are few general reviews on adulteration. Jorgensen

(6) summarized the literature on orange juice, and Mears and Shenton (3) reviewed methods of characterization of orange and grapefruit juices. This chapter reviews the physical, chemical and biological methods of detecting adulterations in citrus products and the reported statistical interpretations of the data. Chapter 18 of this book relates the work of Petrus and Dougherty (7,8) to the detection of pulpwash in Florida FC0J.

Physical Methods

Physical methods directly related to chemical composition have been used in efforts to detect adulterations of citrus products. Physical measurements include spectrophotometry (UV, fluorescence, colorimetry), gravimetric determinations (ash, specific gravity), mass spectrometry (isotope ratios), chromatography, and optical rotation.

Chromatography. Chromatography in its various forms has been a useful tool for determining the composition of citrus products and detecting their adulteration. Examples include the early paper chromatographic work of Rockland et al. (9) and Rockland and Underwood (10) which led to the identification and estimation of the amino acids. Ion-exchange chromatography has been an invaluable tool in the quantitation of the amino acids. Thin-layer chromatography (TLC) by Stanley and Vannier (11,12) helped to establish the identity of the coumarins and psoralins. Column chromatography was used by Horowitz (13,14) and Horowitz and Gentili (15,16,17,18) to separate and identify the flavonoids. Gas-liquid chromatography (GLC) enabled Stanley et al. (19) and Ikeda et al. (20) to determine the volatiles in citrus oils. Although some of the early paper and TLC methods are still used, the new high performance liquid chromatographic and GLC techniques have largely replaced the classical chromatographic approaches.

Isotope Ratios. Doner and White (21) used the isotope-ratio technique to detect adulteration with certain sugars. The technique is based on small differences in the proportions of ^{12}C and ^{13}C incorporated in the various biosynthetic pathways of different plants. Two photosynthetic pathways of the so-called C-3 and C-4 plants provide the basic differences in the $^{12}\text{C}:^{13}\text{C}$ ratio in the sugars of the two groups. Sugar cane and corn both belong to the C-4 group whereas citrus belongs to the C-3 group. As a result, sucrose from cane or glucose or fructose from corn can be readily differentiated by differences in the $^{12}\text{C}:^{13}\text{C}$ ratios from the natural sugars of citrus. However, Nissenbaum et al. (22) pointed out that sucrose from sugar beets has the same ratio as citrus sugars.

Bricout and Mouaze (23) and Bricout et al. (24) demonstrated that hydrogen:deuterium and $^{16}\text{O}:^{18}\text{O}$ ratios differ between juices

and natural waters. The juice contains a higher proportion of ^{18}O and deuterium than does rainwater. This fact could be useful in detecting a reconstituted juice which was claimed to be a natural whole orange juice.

Optical Rotation. Standards established by the Pharmacopeia of the United States (25) require the optical rotation of lemon oils to fall within the range of 57 to 65.5°. Authentic lemon oils, however, have optical rotations that may vary from below 50 to above 70°. Stanley *et al.* (26) demonstrated a relationship between the optical rotation of lemon oil and the hydrocarbon composition. They found a high positive correlation of optical rotation with d-limonene, and a high negative correlation with beta-pinene.

Visible and Ultraviolet Absorption Spectrometry. Born (27) suggested an ultraviolet method for detecting orange peel in orange drinks based on absorbance at 325 nm of a sample extract. Hendrickson *et al.* (28) investigated the determination of hesperidin in orange juice and peel extracts by ultraviolet absorption at 286 nm. They also noted a secondary peak at 330 nm. Although adulteration was not the object of the paper, they concluded orange pulp wash had a much higher overall absorption and a more pronounced peak near 286 nm than did orange juice. Therefore, the addition of pulp washing extracts to an orange juice could be expected to increase the overall absorbance level.

Sale (29) investigated the characteristic ultraviolet absorption of lemon oil and concluded that adulteration of lemon oil with distilled lemon oil could be detected. He recommended other chemical and physical criteria of purity also be considered.

Vandercook and Rolle (30) investigated the total polyphenolic content of lemon juices. Absorption maxima were observed at 326-332 nm and 273-277 nm. They reported that the ratio of the absorbance at 273 nm to that at 326 nm was essentially constant for lemon juice. From analyses of the ultraviolet absorption spectra of grapefruit, orange, and apple juices, addition of such juices to lemon juice could be detected by a displacement of the 273:326 nm absorbance ratio.

Petrus and Dougherty (7) investigated the combined visible and ultraviolet absorption characteristics of alcoholic solutions of Florida Hamlin, Pineapple and Valencia orange juices. Shoulders or peaks were observed at 465, 443 and 425 nm of the visible spectrum (due mainly to the carotenoids present) and 325, 280 and 245 nm of the ultraviolet spectrum (due mainly to polyphenols, flavonoids and ascorbic acid, respectively). Absorbance of the maxima peaks obeyed Beer's law while the 443:325 nm absorbance ratios remained essentially constant. When fruit extractor pressures were increased, the UV absorbance of the resulting juice increased and the 443:325 nm absorbance ratio decreased. Spectra of alcoholic solutions of the rag and albedo components

of oranges showed little visible but strong UV absorbance. Petrus and Dougherty (8) also investigated the spectral characteristics of mid- and late season frozen orange concentrates and corresponding frozen orange pulpwash concentrates obtained from commercial processors. Alcoholic solutions of orange pulpwash samples were characterized by weak visible and very strong ultraviolet absorption. Weak visible absorption was due to traces of parent orange juice. The strong ultraviolet absorption was due to the presence of the parent orange juice plus soluble solids extracted from rag and pulp during the pulp washing process. The parent orange juice concentration in the pulpwash product was estimated from the absorbance ratio at 443 nm of pulpwash to orange juice.

Luminescence spectrophotometry consists of fluorescence, phosphorescence and low-temperature total luminescence. Fluorescence is generally measured at room temperature. Phosphorescence is generally observed at liquid nitrogen temperature (77K) with the aid of a chopper to interrupt the exciting radiation. Total luminescence is the combined fluorescence and phosphorescence obtained at low temperature (77K). Luminescence spectrophotometry is generally much more sensitive and specific than absorption spectrophotometry.

Vannier and Stanley (31) employed fluorescence spectrophotometry to analyze mixtures of grapefruit and lemon oils. D'Amore and Corigliano (32) used fluorescence to characterize mandarin, lemon and bergamot oils and related the characteristics to detect adulteration of mandarin oil with terpenes.

Latz and Madsen (33) investigated the total luminescence of coumarin derivatives isolated from expressed lime oil. They suggested the possibility of using excitation and emission spectra to detect the presence of chalcones and methyl salicylates added to adulterate expressed oils.

Chapter 18 reports the investigations of Petrus and relates the room temperature fluorescence, and visible and UV spectral characteristics of citrus juices and related products to the detection of adulteration.

Ash and Mineral Analyses. Many classical methods for estimating fruit content in foods and beverages were based upon determining inorganic constituents, such as total ash and the alkalinity of the ash. The procedures were refined by Morgan (34) to correct for inorganic sulfites, benzoates and phosphates added as preservatives or adulterants. The classical methods in many cases have been replaced by spectrographic procedures for the determination of individual elements. McHard et al. (35) conducted a comparison study of metal constituents in orange juice by flame atomic absorption spectrometry, flame atomic emission/fluorescence spectrometry, direct current plasma atomic emission spectrometry, and inductively coupled plasma atomic emission spectrometry. They observed differences between methods with

regards to speed, convenience, precision, detection limits, and the interpretation of calibration curves but found all spectral methods in general agreement.

Brix. Two methods generally are used to measure Brix or the soluble solids of citrus products. A hydrometer is used to determine the solution specific gravity and a refractometer to determine the solution refractive index (see Chapter 13).

The system of hydrometer graduation was devised by Balling (36) and recalculated by Brix (37). Antoine Baume (38), a French chemist, devised the Baume hydrometer in 1768, and the readings are known as degrees Baume. In the United States the hydrometer has become known as the Brix hydrometer, and the readings are in "degrees Brix". The degree Brix is the weight percent of sucrose in a pure solution. The Brix of an impure sugar solution (citrus juices may be considered an impure sugar solution) is higher than the weight of solids obtained by drying because non-sugars generally have a higher density than sugars.

The refractive index of a pure sucrose solution is an accurate measure of the concentration of dissolved substance. Stolle (39) found little variation in the refractive index of solutions of different sugars (sucrose, dextrose, levulose and lactose) at the same concentration. Investigations by Main (40), Schonrock (41) and Landt (42) resulted in the adoption of a standard table of refractive indices of sugar solutions by the International Commission for Uniform Methods of Sugar Analysis in 1936.

The determination of degrees Brix by floating hydrometer or refractometer is an important measurement for the citrus industry. The Brix is used in connection with the acidity as a measure of citrus maturity and quality. The degrees Brix is also used in commerce as a juice concentration standard as exemplified by the Florida Department of Citrus rule which requires frozen concentrated orange juice to be 44.8° Brix which reconstitutes to 12.8° Brix (43). The U. S. standards require 41.8° Brix which reconstitutes to 11.8° Brix (44). Even though degrees Brix is an important determinant it is of little value for detecting dilution because of the ease of adding sugar products to disguise the adulteration.

Chemical Methods

Many chemical methods have been tested and proposed for detecting adulteration. Some methods are non-specific and measure a general class of compounds whereas others are specific for a given compound. Each type has advantages and disadvantages in terms of analytical time, economy, difficulty and ease of circumvention. The various classes of citrus constituents will be discussed separately.

Amino Acids. The amino acids are an important group of com-

pounds which have received a great deal of attention. The first indirect use of free amino acids to detect adulteration was by Tillmans and Kiesgen (45) through the use of the formol index (F.I.). Over the years the method has become a widely used parameter in estimating juice content or adulteration. Typical values are shown in Table I.

Other methods of estimating total amino acids exist but have not been widely applied to citrus. Various ninhydrin colorimetric methods are published, but different colors produced by individual amino acids caused quantitation problems. Ting and Deszyck (63) attempted to reduce this problem by using two wavelengths, 400 and 570 nm, for proline and the "ninhydrin blue" amino acids. Differences in molar absorption made the results dependent upon the composition of the standard mixture.

Vandercook et al. (64) developed an automated ninhydrin procedure and measured the absorbance at 480 nm where the molar absorbances of the major orange juice amino acids are nearly equal. Analysis of a series of synthetic amino acid mixtures representing the extremes in amino acid composition (46) resulted in a maximum error of 5%.

Hils (65) applied a trinitrobenzene sulfonic acid method to determine the amino nitrogen of orange juice, and found an average of 20 and 23 mg amino N/100 ml juice for laboratory-prepared and commercial orange juice, respectively. Benk and Krause (66) applied the method to both laboratory-prepared and commercial orange, lemon, and grapefruit juices. They found higher values for fresh orange juice than Hils reported and similar values for commercial juice, 31 ± 6 and 19 ± 3 mg amino N/100 ml juice, respectively. Fresh and commercial grapefruit juice had values of 18 ± 3 and 17 ± 3 , respectively.

Unfortunately, there are numerous ways to circumvent the analysis of total amino acids, such as by the addition of ammonium salts, inexpensive amino acids, peptides and protein hydrolysates. Several approaches have been made to verify the authenticity of the total amino acid values. Rockland and Underwood (10) developed a paper chromatographic technique for quantitatively estimating the individual amino acids.

Adjustment of the formol values in commercial orange juice with inexpensive amino acids such as glycine has been detected by Benk and Cutka (67) by means of thin-layer and paper chromatography. Aranda et al. (68) studied the TLC amino acid patterns as a means of detecting adulteration by amino acids and concluded that amino acids added in sufficient quantities to balance the formol index value could be detected.

Royo Iranzo and Cervello (69) suggested using the ratio of total N to amino N to verify the authenticity of orange juice. For 70 Spanish orange juices they reported a ratio of 2.98 ± 0.22 mg total N/mg amino acid N. Added amino acids with a single N would have a ratio of approximately 1.0 (depending upon formol response) and would lower the natural ratio of the juice. How-

Table I. Representative Formol Values (meg/100ml) of Citrus from Various Sources^a

Variety	California	Florida	New Zealand	Italy	Israel	Spain	Greece
Orange (single strength or calculated to 11°Brix)	2.17 ± .36	1.77 2.04 1.63 ± .39	3.00 ± .52	1.01-2.56 1.46-1.95	1.51 ± .42 1.27 ± .13	1.48 3.16 ± .61	1.75 (0.58-3.74)
Lemon (single strength or calculated to 6% anhyd. citric acid)	2.13 ± .33	1.72 ± .08 (Bears) 1.95 ± .08 (Meyer)	1.65 ± .35 2.00 ± .32 (Meyer)	1.00-2.10 0.60-1.80 1.20-2.46 1.65 ± .28	2.09 ± .49		
Grapefruit	1.92 (1.64-2.27)	2.04	2.05 ± .67 2.50 ± .57 (Wheeny)	1.9	2.1		

^aData compiled from various sources. References by variety and origin. Orange: California (46); Florida (46, 47, 48); New Zealand (49); Italy (50, 51); Israel (52, 53); Spain (53, 54); Greece (55). Lemon: California (46); Florida (46); New Zealand (56); Italy (46, 57, 58, 59); Israel (60). Grapefruit: California (46); Florida (46); New Zealand (49); Italy (61); Israel (62).

ever, amino acids with more than one N such as asparagine or arginine would have ratios of approximately 2.0 or 4.0, respectively, and would be less likely to be detected by this method.

Wallrauch (70) suggested a different approach to detecting altered amino acid values. Since proline (without the primary amino group) does not react with formaldehyde, the ratio of proline to the formol number is a potentially useful indicator of adulteration. The average ratio was found to be 19 with a standard deviation of 5.

Several workers reported finding commercial samples of orange juice in which the formol value had been adjusted with ammonium salts (67,71,72). These workers suggested the determination of ammonia as a means of detecting this type of adulteration.

Gierschner and Baumann (73) suggested the possibility of detecting peel constituents, added to extend orange juice, by measuring the formol value on filtered and whole juice. The whole juice has a higher formol value which increases with the amount of added peel, whereas the formol value of filtered juice remains constant. The problem of peel extracts being added to extend a juice was pointed out by Green and Wells (74) when they examined peel, rag and juice fractions of Sicilian Lemons. They suggested the formol value was not practical in examining comminuted fruit beverages since the level of amino acids was significantly higher in the peel. However, Wucherpfennig and Franke (75) in a study of the free amino acids of orange juice and peel failed to find any significant differences in formol values of the peel and juice.

The development of the commercial amino acid analyzer based on the ion-exchange method led to the rapid accumulation of compositional data on the amino acids in citrus. Many investigators have published amino acid compositional data. A summary from a recent review (76) is presented in Table II. These data make it relatively easy to detect adulterations with amino acids, although the equipment and analyses are expensive for routine testing.

Various workers have sought to simplify the verification of the total amino acid estimates by measuring some of the major amino acids and relating these values to the total.

Arginine and γ -aminobutyric acid contents of orange juices were studied over a period of several years by Vandercook and Price (80) and by Vandercook et al. (64). It was suggested that these values, plus their ratios with other constituents, such as amino acids, phenolics and sugars, would be helpful in detecting adulterations of orange juice. Values for several seasons of Florida, California, Arizona and Mexican orange juice are presented in Table III.

Morgan (81) proposed serine concentration as a possible index of orange juice content. He found serine to be uniformly distributed between the peel, pulp and juice. For orange juices

Table II. Amino Acid Composition (mg/100ml) of Citrus Juices from Various Sources^a

<u>Amino Acids</u>	<u>Orange</u>	<u>Lemon</u>	<u>Grapefruit</u>	<u>Tangerine</u>
Arginine	31 - 72	2 - 7	16 - 47	19 - 84
Ammonia	0.9 - 2	3 - 5	2	1
Lysine	3 - 7	1	0.8 - 3	4
Histidine	0.4 - 2	0.6 - 1	0.2	-
γ-Amino- butyric acid	14 - 50	7 - 16	8 - 19	14 - 18
Phenylalanine	1 - 3	1 - 3	0.7 - 3	5
Tyrosine	0.9 - 3	0.2 - 0.8	0.3	-
Leucine	0.4 - 1	0.7 - 1	0.3	3
Isoleucine	0.5 - 0.9	0.8 - 0.9	0.3	-
Methionine	0.1 - 0.9	0.3 - 0.7	0.1	-
Valine	1 - 3	1 - 3	1 - 2	2
Alanine	8 - 12	10 - 23	7 - 9	7 - 11
Glycine	1 - 2	1 - 2	0.4 - 2	2
Proline	67 - 239	29 - 84	41 - 59	38 - 100
Glutamine	1 - 4	1	3	-
Glutamic acid	7 - 18	16 - 31	18 - 22	16
Asparagine	16 - 50	16 - 28	15 - 42	4 - 85
Serine	9 - 22	18 - 47	10 - 15	19 - 22
Threonine	1 - 2	1	1	4
Aspartic acid	16 - 41	34 - 65	34 - 81	14 - 36

^aData compiled from various sources. References by variety and origin. Orange: California (46, 77); Florida (46, 78); Italy (61); Greece (55). Lemon: California (46, 77); Florida (46); Italy (79); Israel (60). Grapefruit: California (46); Italy (61). Tangerine: California (77).

Table III. Concentrations of γ -Aminobutyric Acid and Arginine (mg/100 ml \pm std. dev.) Orange Juices (Reconstituted to Single Strength) (80)

Source	Season	No. of samples	γ -Aminobutyric acid	Arginine
Calif.	1969-70	7	50 \pm 20	77 \pm 22
Calif.	1970-71	13	55 \pm 6	96 \pm 23
Calif. Valencia	1971	8	45 \pm 7	136 \pm 10
" "	1972	17	40 \pm 10	117 \pm 26
" "	1973	20	36 \pm 8	98 \pm 28
Calif. Navel	1972	6	33 \pm 5	52 \pm 12
" "	1973	4	30 \pm 3	71 \pm 12
Florida	Early 1971-72	4	26 \pm 4	48 \pm 7
	Mid "	12	29 \pm 4	47 \pm 12
	Late "	18	59 \pm 8	90 \pm 19
	Early 1972-73	2	21 \pm 3	52 \pm 5
	Mid "	19	33 \pm 8	78 \pm 16
	Late "	6	29 \pm 2	68 \pm 5
Florida (pulp-wash)	Mid 1971-72	12	57 \pm 19	47 \pm 10
	Late "	18	59 \pm 12	74 \pm 17
	Mid 1972-73	13	34 \pm 7	59 \pm 14
Arizona	1972-73	12	35 \pm 8	54 \pm 9
Mexico	1972	10	26 \pm 6	87 \pm 12
	1973	19	29 \pm 5	78 \pm 14
Overall Mean			38 \pm 12	75 \pm 25

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and comminuted products from 5 countries he calculated the overall serine mean concentration to be 19.2 mg per 100 g. The method is rather complicated (filtration, extraction, ion-exchange, concentration, reaction with periodate, distillation and final colorimetric reaction) and has not been widely used.

Lewis (82) proposed the use of betaine as an indicator of orange juice content in compounded orange beverages. By means of ion-exchange he determined the average value of betaine to be 72.4 mg per 100 ml in orange juice (11.0% solids). He found betaine rather constant with a standard deviation of 4.5 and coefficient of variation of 6.2%. A collaborative study was undertaken by Rogers (83) to evaluate the procedure for the Association of Official Analytical Chemists. The method was subsequently adopted as official by the AOAC (84). Coffin (85) applied the method of Lewis to orange juices obtained from Canadian market sources. He reported a lower mean and larger standard deviation (54.6 ± 13.6 mg per 100 ml). Florida orange concentrates were reported to have an average betaine content of 66.8 ± 8.3 mg per 100 ml at 11.8% soluble solids (86).

Various combinations of amino acids have been proposed as a means of determining authenticity. Zamorani *et al.* (87) and Russo *et al.* (88) suggested the ratio of (proline + arginine): (aspartic acid + asparagine). Due to concentration changes with maturation and season, they claimed that the ratio of these four amino acids is more constant and characteristic of the citrus variety than is the absolute concentration. They reported that the ratio is around 3 for oranges and tangerines, 1 for grapefruit, and 0.3 for lemons. Habegger and Sulser (89) used the sum of all the free amino acids and the sum of serine, proline, γ -aminobutyric acid and arginine along with the ratios of these sums to characterize the orange juice.

Other investigators have used maximum and minimum concentration values of specific amino acids as a criteria of authenticity. Brenoe (55) reported means and confidence intervals for all of the amino acids in Greek orange juice. Special emphasis was placed on glycine since it is present in very low levels in citrus juices but is relatively abundant in protein hydrolysates.

Niedmann (90) considered the amino acid composition of orange juices and hydrolysates of commercial proteins. Based on the statistical distributions he proposed the following minimum values for the six major amino acids for normal orange juice: proline, 5 mmol/l; arginine, 2; asparagine, 1.75; γ -aminobutyric acid, 1.5; aspartic acid, 1.5; and serine, 0.7. Koch (91) proposed a set of maximum values for glycine and glutamine, 0.5 and 0.1 mmol/l, respectively, based on literature values and his own analyses. In addition to the minimum values suggested by Niedmann he proposed minimum values for glutamic acid and alanine of 0.5 mmol/l.

It was suggested by Gherardi *et al.* (92) that adulteration of orange juices with pulp and peel extracts could be detected by

the levels of the minor amino acids: valine, leucine, isoleucine, methionine, tyrosine and phenylalanine. These amino acids comprised a total of 1.5% in natural juice and 3.7% in pulp and peel extracts.

Organic Acids. Organic acids are an important part of citrus juices from an organoleptic point of view. They have also been used both to detect adulterations and as the adulterants. Lemon juice for example is sold on the basis of its acidity, which is primarily citric acid. Adulteration with added citric acid has been a long-standing problem. Sometimes citric acid is added along with sugars to orange juice to maintain the proper Brix-acid ratio.

The acid present in the next largest amount is l-malic acid. Vandercook et al. (93) used l-malic acid as determined by optical rotation, along with other constituents as a means of detecting adulterations in lemon juice. The acid concentration shows a wide range of values in lemons and decreases dramatically with fruit storage (94). The low cost of d,l-malic acid and the high cost of l-malic acid influences the selection of analytical methods when using this parameter to detect adulteration. If only total malic acid was determined, adulteration could be inexpensively disguised with added d,l-malic acid. The enzymatic method which measures the levo form could also be circumvented by adding twice the amount of d,l-malic acid. Thus to substantiate the enzymatic method, a determination of total malic acid would also be necessary (95,96). The AOAC (97) optical rotation method, on the other hand, would not be influenced by added d,l-malic acid.

Primo Yufera et al. (98) looked at the organic acids in orange juice by thin-layer chromatography as a qualitative technique for detecting adulterations. Primo Yufera and Royo Iranzo (99) studied the neutralization characteristics of lemon and orange juices and concluded that the change in pH with added base could be used to detect citric acid added to these juices. The change appeared to be due to differences in the buffering capacity of the juice and a citric acid solution. No data was presented on the effects of adulteration with both citric acid and potassium citrate.

The same investigators qualitatively examined the organic acids in orange juice by GLC (100). They reported the following acids: lactic, oxalic, malonic, phosphoric, succinic, benzoic, adipic, malic, tartaric, iso-citric, aconitic and citric. They (100,101) also examined commercial sucrose and citric acid in an attempt to detect acidic impurities in these adulterants. Differences in the acid patterns were observed, but they concluded that the levels in sugar and citric acid were too low to be useful in detecting adulterations.

Recently, isocitric acid has been successfully used to detect adulterations with citric acid and simple dilutions (102,103

104,105,106,107,108,109). The acid is measured using the enzyme isocitrate dehydrogenase. Isocitrate is present in a small but fairly constant amount. Table IV gives the concentrations and ratios with citric acid.

Bergner-Lang (104) reported that 74% of 80 commercial orange juice samples tested failed to meet the citrate-to-isocitrate ratio of a series of laboratory-prepared orange juices, and 87% of the same samples were below the minimum for fresh juice. These conclusions were challenged by Rother (108) as inappropriate due to the high percentage of Brazilian orange juice in the commercial products. He presented data where 17 of 19 samples fell within the normal citrate-to-isocitrate ratio range of 50 to 170.

The procedure was recently automated by Buslig and Ting (110). Their values for Florida orange juice were close to the published values and did not show any significant changes due to juicing and finishing methods.

Since isocitric acid is quite expensive, adjusting it to the normal range in an adulterated juice would not be economically practical. The price situation could change, however, if heavy emphasis is placed on this acid as a criterion of authenticity. At least two patents have been issued for production of the acid by fermentation processes (111,112).

Phenolics. The phenolics of citrus represent a varied and widely studied class of compounds. They range from the lipid-soluble methoxylated coumarins and psoralens to the water-soluble glycosides of the flavanones and flavones. They include the intensely bitter naringin and the highly insoluble hesperidin. Different classes within the group have characteristic UV spectra which have been used to detect adulterations in juices and oils.

One widely used estimate of phenolic compounds is the Davis test (113). In the presence of base the UV spectrum undergoes a bathochromic shift, and a yellow color is produced. The values are commonly expressed as hesperidin for orange or lemon juice or naringin in the case of grapefruit. Davis found higher flavanone values from the albedo, segment membrane, core and flavedo than from the juice vesicles. Rouse *et al.* (114) found more flavonoids in water extracts of orange pulp than in the orange juices collected from the same processors at the same time. Horowitz and Gentili (115) pointed out some of the limitations of the Davis method, but probably due to simplicity the method is still very popular. A proposed alternative to the Davis test was made by Hendrickson *et al.* (28). Hesperidin is estimated in the juice by UV absorbance after a treatment with copper sulfate and aeration.

Another parameter useful in detecting adulteration is total phenolics as estimated by UV absorbance at a standard dilution (7,8,30). No attempt was made to relate the absorbance to the concentration of any specific compound. The absorbance and the

Table IV. Isocitrate Concentrations and Ratios with Citrate in Citrus Juices

Juice	Isocitrate		Citrate/Isocitrate		Ref.
	Mean	Std. Dev.	Mean	Std. Dev.	
Lemon	0.219 ± .047	0.161 - .365	236 ± 32	162 - 285	(109)
	0.302 ± .050	0.240 - .424	215 ± 24	174 - 258	(103)
Orange	0.131 ± .060	0.044 - .247	109 ± 34	56 - 168	(102)
	0.108 ± .030	0.081 - .175	135 ± 9	122 - 158	(104)
Grapefruit	0.136 ± .024	0.100 - .204	156 ± 30	120 - 258	(110)
	0.145 ± .030	0.055 - .204	130 ± 45	80 - 228	(102)
Satsuma	0.128 ± .032	0.060 - .191	116 ± 24	57 - 169	(110)
	0.080 ± .007	0.070 - .086	126 ± 9	119 - 140	(103)
Clementine	0.025 ± .003	0.020 - .029	199 ± 32	170 - 243	(103)

shape of the UV spectra were proposed to characterize lemon juice (116). Before the juice shows visible signs of browning, the UV spectra in the region of 310 to 332 nm changes. The peak at 332 nm for fresh juice shifts to a shorter wavelength as the juice deteriorates. Likewise, the minimum at 310 shifts to longer wavelengths and becomes less distinct until it finally disappears in a juice which has begun to turn brown.

An estimation method for total phenolics in orange juice was automated by Vandercook *et al.* (64). In this procedure a diazotized sulfanilic acid was coupled with the phenolics of the juice. The reaction was calibrated against phenol as the standard.

One of the earliest uses of phenolic compounds to detect adulterations was in lemon oil. Vannier and Stanley (117) developed a procedure to detect grapefruit oil (which is relatively cheap) added to the more expensive lemon oil by detecting 7-geranoxycoumarin. By thin-layer chromatography, hydrolysis, and fluorescence in basic media, as little as 2% grapefruit oil in lemon oil could be detected. Stanley (118) also used the coumarins and psoralens to detect adulteration of cold-pressed lemon oil with cheaper distilled lemon oil plus chalcones. The spectra of the chalcones was similar to that of the cold-pressed oil, although lemon oil contained no chalcones. Presence of the adulterant was determined by thin-layer chromatography.

Paper chromatography was used to estimate individual phenolics in lemon juice by Vandercook and Stephenson (119). After enzymatic hydrolysis of the juice, they found for eriodictyol, hesperitin, quercetin, phloroglucinol and umbelliferone average values of 20, 1.4, 2.2, 2.0 and 0.2 mg aglycone/100 ml, respectively.

Gas-liquid chromatography was used by Coffin and DuPont (120) to separate the trimethylsilyl derivatives of the flavanones. Quantitation and resolution of the compounds presented some problems, and only one or two of the major flavonoids could be determined.

Several TLC methods have been widely used to quantitatively estimate the flavonoids for quality control purposes rather than to detect adulteration. The potential exists, however, for testing authenticity. Naringin is an important compound in grapefruit juice, since it is largely responsible for the bitter character of the juice. Fisher *et al.* (121) developed a TLC procedure for naringin estimation. This was later modified by Tatum and Berry (122). Swift (123) developed a TLC-spectrophotometric assay for the neutral methoxylated flavones in orange peel. The method was subsequently expanded to the determination of these compounds in orange juice (124).

An extraction procedure and UV measurement of hesperidin was proposed by de la Torre Boronat *et al.* (125). They proposed the method as a means of determining the amount of juice in orange based drinks.

Several investigators have used HPLC to analyze for the individual flavonoids in juices. Fisher and Wheaton (126) used a reverse phase μ -Bondapak C-18 (Waters Associates) column to separate and quantify naringin and naringenin rutinoside. Bennett and Schuster (127) applied a similar reversed phase system to the measurement of the flavanones in lemon juice.

Chloramine-T. The chloramine-T determination (128) has been used as a test for dilution or adulteration of citrus juices. The chloramine value is a measure of the reducing constituents present in citrus juices other than sugars and acids (129,130).

Maraulja and Dougherty (131) reported chloramine-T values for Florida Hamlin, Pineapple and Valencia were slightly higher for hard squeeze juices than for soft squeeze juices. Chloramine values were considerably higher for the albedo and flavedo components than for juice and were suggested for detecting adulteration with water extracts of peel and rag (75,132). Chloramine values are often included with other analytical parameters for citrus juice authentication (133,134).

Sugars. The soluble solids of oranges, tangerines and grapefruit juices consist mostly of sugars, whereas soluble solids of mature lemon and lime are mainly composed of citric acid. Floyd and Rogers (135) reported no significant changes, due to juice concentration, in the composition or distribution of sugars in orange juice. Sucrose, glucose and fructose, in a ratio of 2:1:1, were found to be the principal sugars in Florida Valencia orange juices (136). Ting (137) developed a rapid colorimetric method for simultaneous determination of total reducing sugars and fructose in citrus juices. Sawyer (53) included total sugars, reducing sugars and sucrose in his chemical analyses for the characterization of fresh and concentrated orange juices and "pulp extracts". Sawyer also included pentose equivalent values (expressed as equivalent xylose content per 100 ml of juice) as an indication of added peel and peel extractives. Benk (138) reported adulteration of citrus juices with rag and peel extracts should be relatively easy to detect because their pentose equivalent values were about three times higher than those obtained for orange juices.

Lipids. Nagy (139) indicated that a knowledge of lipid content and distribution in citrus products of commercial origin might be useful in detecting adulteration. He reported that the concentration of specific lipid components in single-strength orange and grapefruit juices might possibly be used to indicate excessive dilution of a reconstituted product. Vandercook *et al.* (140) qualitatively and quantitatively investigated the major phospholipids of orange, lemon and grapefruit juices and suggested that the values may be used to distinguish juices adulterated with commercial lipids used as clouding agents or emulsifiers.

Vitamins. Some carotenoid pigments in citrus have provitamin A activity. Besides the nutritional aspects, the carotenoid color of citrus products has a profound effect on consumer acceptance. Therefore, pigments, such as β -carotene, are sometimes added to improve a poorly colored or diluted product. Many methods have been developed or adapted for the determination of natural and/or added colorants of citrus juices (141,142,143,144,145). The determination of β -carotene is often included with analytical parameters in testing for juice purity. Higby (146) concluded that finding more than 10% carotenes in the total carotenoids of an orange juice indicated addition of β -carotene. Estimation of cryptoxanthin content, which is higher in mandarin than in orange juices, was suggested by Koch and Haase-Sojak (147) to detect addition of mandarin juice.

Sawyer (53) included the concentrations of ascorbic and nicotinic acids along with other analytical parameters to detect orange juice adulteration by dilution or addition of peel extracts. He observed that the relation between the total invert sugar to total soluble solids ratio and nicotinic acid became significantly correlated by the dilution of juices with sucrose or partially inverted sucrose.

Lisle (148) investigated the inositol and nicotinic acid content of orange juice samples from Israel, South Africa, British Honduras and Southern Rhodesia. From the analytical results the equation

$$C = 2.20X + 0.0025Y$$

was derived for the estimation of orange juice content of concentrate, where C is the concentration in units of 10⁰ Brix, X is the nicotinic acid content (mg/100 ml), and Y is the inositol content (mg/100 ml).

Minerals. Determinations of inorganic constituents (e.g. total ash, alkalinity of the ash) are classical methods for estimating juice content of citrus juices and beverages. Refinements were made to correct for inorganic sulfites, benzoates and phosphates used as adulterants or preservatives (34).

Mineral profiles of processed juices are dependent on many variables: growing conditions (fertilization, rainfall, soil, climate, root stock, geographical origin, variety and maturity), processing conditions (type of extractor and setting, finisher setting, condition of fruit, juice yield), and contamination (phosphate-containing detergents, alkali solutions for cleaning equipment, fruit washing solutions). In addition to the uncertainty caused by the natural and processing variables professional adulterators use "tailor made" adulterants with the major inorganic elements in about the correct proportions.

Hulme *et al.* (149) proposed the equation $0.05(7K + 10P + 3N)$ to estimate the fruit content of beverages made from whole

oranges, where K, P and N are the analyses values for potassium, phosphorus and nitrogen, respectively. Any or all of these elements may easily be adjusted.

Vandercook and Guerrero (150) characterized orange juices by fractionating the total phosphorus content (19.3 ± 2.5 mg P/100 ml) into lipid (1.4 ± 0.3), inorganic (12.6 ± 2.9), and ethanol-insoluble (3.0 ± 0.8) phosphorus. A limited number of grapefruit and lemon juices were also characterized.

Royo Iranzo and Garcia (151) suggested that analyses for juice characterization and detection of adulteration be conducted on the serum. They reported that sodium, calcium and phosphorus were all higher in the pulp than in the serum. The values for magnesium were about the same for pulp and serum; potassium was slightly higher in the serum than in the pulp.

McHard et al. (152) using plasma spectroscopy investigated 32 elements in Florida and Brazilian frozen concentrated orange juice samples. Using probability statistics they suggested the relative occurrence of the minor elements Ba, B, Ga, Mn and Rb as ratios to zinc could act as "fingerprint" indicators of the geographic source of a sample. No relationship to adulteration was implied.

Volatiles. The volatile components present in citrus fruits are very important factors contributing to the characteristic flavor and aroma of the fruits and juices but are of minor importance to detecting adulteration in the juice. Swift (153) devised a GLC method for the determination of linalool and alpha-terpineol in orange juices and oils. It was implied that some extraction procedures might affect the linalool value. In concentrated products, both compounds would be removed with the water vapor. Volatiles found in the final product are generally the result of added cutback juice or added oil. However, Murdock et al. (154) reported that alpha-terpineol may be increased by bacterial action during processing of orange juice.

D'Amore and Corigliano (155) indicated the intense fluorescence of Mediterranean mandarin oil at 415 nm might be useful for characterization and detection of adulteration. Slater (156) reported the ratio of some infrared peak intensities might be useful for detection of adulteration or storage abuse in citrus oils.

The Pharmacopeia and the Essential Oil Association of the United States require the following analyses as an index of oil purity; refractive index, optical rotation, refractive index and optical rotation of a 10% distillate, specific gravity, aldehyde content, evaporation residue and ultraviolet absorption spectrum (Kesterson et al. 157 and Sale 29).

Biological Assays

Biological assays have a significant potential to detect adulterations but have been generally overlooked for this appli-

cation. Cantagalli *et al.* (158) proposed an immune assay for orange juice using rabbit serum. They isolated protein fractions from orange juice and used it to prepare rabbit immunosera. The test distinguished lemon juice from orange juice, was sensitive to commercial orange juice products, and was insensitive to common food additives. Firon *et al.* (159) used the immune assay for estimating juice content of orange juice drinks in the 10% juice range.

Another bioassay recently reported by Vandercook *et al.* (160, 161) involves the use of *L. plantarum*, a fastidious organism which requires many nutrients for growth. Growth of the organism in the orange juice assay system is proportional to the amount of juice present. Thus, its growth is an indication of a complex array of nutrients in the juice. The first growth-limiting nutrients for the organism in orange juice are the amino acids leucine, isoleucine, valine and methionine (162). When an amino acid supplement is added to the assay mixture enhanced growth occurs. The second growth-limiting nutrient is the mineral manganese. A further growth enhancement occurs when an amino acid-manganese mixture is added to the assay system. The bacterial growth response of orange juice, orange juice plus amino acids, and orange juice plus amino acids and manganese (7.7, 26, and 63 absorbance relative to a standard, respectively) provide a potentially useful set of parameters for detecting adulterations.

Automation of a modified version of this assay was recently reported by Vandercook *et al.* (163). In the automated system, each sample is inoculated with a massive number of cells. The rate of pH change is followed automatically for a standard and the samples. The rate of pH change is proportional to the concentration of orange juice in each sample. Ten samples can be assayed in triplicate in 80 min. Data from 72 retail FCQJ samples compared with a composite sample of authentic orange juice showed an average estimated orange juice content of 97% and standard deviation of 11.5%.

Data Evaluations

Experimental data obtained by any of the assay methods must be evaluated by someone. Judgements are commonly based on the experience of the analyst and accumulated laboratory data or published results. The evaluations range from comparison with a simultaneous standard to highly sophisticated statistical equations requiring many calculations. Evaluation of juice content should be considered as an estimate in the context of placing the sample somewhere in the natural population distribution, and the probability of that estimate should be reported. Unfortunately, many literature reports fail to mention or minimize the uncertainty of the estimate. In samples where the presence of foreign substances is proven, one can state with absolute certainty that the juice has been adulterated.

Most researchers agree that measurements of several constituents give better estimates of juice content than a single parameter. The question of how many and which constituents is open to debate. Furthermore, an optimum statistical treatment of the data has not been agreed upon.

Equations based on multiple regression of several constituents have been proposed by a number of workers. Rolle and Vandercook (164) suggested such an approach to estimate citric acid in lemon juice from the values of total amino acids, l-malic acid and total phenolics. Coffin (85) examined commercial orange juice for total amino acids, betaine, polyphenolics, ash and phosphorus. He found several significant correlations and derived a multiple regression equation relating total amino acids to the other constituents. Vandercook *et al.* (165) revised their original lemon juice multiple regression equation to eliminate concentration effects and to consider the effects of multiple samples on estimation accuracy and error probabilities. The equation is $CA/TP = 10.22 + 31.06(AA/TP) + 6.36(MA/TP)$ where CA = citric acid, TP = total phenolics, AA = amino acids, and MA = malic acid. For example, they reported that with five representative samples of juice from a given processor a 14% adulteration with citric acid could be detected 90% of the time with a 95% level of confidence.

Lifshitz *et al.* (166) proposed a chi-square (χ^2) test for detecting adulteration in lemon juice which, for their data, is more sensitive to dilution than the multiple regression approach. The same group (133) used a multivariate normal test on five parameters (Brix, formol number, chloramine-T number, total sugars and chlorides) of Israeli orange and grapefruit juices. Based on their data an adulteration of 15% or more was "very likely to be detected at the 1% level of significance".

Fischer (167) proposed groups of constituent ratios as a means of evaluating orange juice. Nine groups of 29 ratios between total sugars, N-compounds, non-volatile acids, ash and ash constituents were used to detect dilutions and various adulterants.

An elaborate and novel system was devised by Richard and Coursin (168,169,170,171) whereby 19 constituents (minerals, sugars, acids, amino acids) were determined and evaluated by a hierarchical classification approach. By means of a series of inequalities, based on deviations from the mean, a region of authentic juice is defined in a multidimensional space. A series of regression equations between parameters (with $R > 0.9$) are considered next to verify that the relationships between constituents are normal. Finally, the above information may be combined in a matrix approach to give an estimate of juice content.

A non-parametric classification technique was recently proposed by Schatzki and Vandercook (172) to evaluate orange juice. The system considers the total sugars, reactive phenols, total amino acids, arginine and γ -aminobutyric acid. With the para-

meters measured, the results showed that detection was possible with a type 1 = type 2 error rate of 10% for a 20% adulteration if at least 7 samples were analyzed. The non-parametric technique in general has an advantage over classical statistics in that it requires no assumptions regarding the normality of the data. The method applied to different parameters might give a more sensitive response to adulteration than the parameters they reported.

A subgroup of the German Chemical Society (173,174) recently published a system which proposed a means of evaluating the juice content of low-juice beverages. It is based on the concentrations of potassium, phosphorus, proline, formol number, isocitric acid and malic acid. Equations for the juice content of lemonades and orange juices (with possible mixture of tangerine or mandarin juice) based drinks are:

$$\% \text{ lemon juice} = 0.0114(K) + 0.05(P) + 0.0267(\text{Proline}) + 0.8333(\text{Formol No.}) + 0.1111(\text{Isocitrate})$$

$$\% \text{ orange juice} = 0.0114(K) + 0.05(P) + 0.0267(\text{Proline}) + 0.8333(\text{Formol No.}) + 0.1111(\text{Isocitrate}) + 0.01(\text{Malate}).$$

The formol number is checked for authenticity by means of its ratios with proline, ammonia, alpha-amino nitrogen, and amino acid analyses. The malic acid is checked by enzyme and chemical assays. Based on their data, the Subgroup considers the system to be accurate enough to detect a 10% difference between the measured and stated juice concentration. Their data indicate good agreement between six cooperating laboratories.

A possible weakness in this system lies in the fact that a substantial fraction of the calculated value of percent juice comes from potassium and phosphorus which would be easy to add.

Conclusions

A great deal of time and effort has gone into the development of methods to detect adulteration in citrus products. Unfortunately, considerable efforts continue to be directed towards means of circumventing adulteration detection techniques. The problem is basically one of economics and probability on both sides. Once a constituent is defined quantitatively it can be added to compensate for dilution. For the adulterator the situation is a matter of balancing the risk of being caught against the cost of adding constituents to avoid detection of a dilution. For the enforcement agencies the problem is a question of balancing the risks of accepting an adulterated product or rejecting an authentic product against the costs of a greater number of analyses to improve the probability of a correct judgement. Furthermore, the problem is a dynamic one over which the scientist has little control. There are political and legal considerations along with the economic factors of supply and demand.

Each situation may be unique and requires a careful balancing of all factors. Continued research will be necessary to more fully understand the many factors affecting the composition of citrus products. One single, simple, inexpensive adulteration test may never be available. The current technology for adulteration detection can be applied, keeping in mind the statistical limitations of the selected parameters. With the current trends towards multiconstituent analyses and an increasing awareness of statistical interpretations, detection of adulteration should become more sensitive and accurate in the future.

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Detection of Adulteration

Visible and Ultraviolet Absorption and Fluorescence Excitation and Emission Characteristics of Florida Orange Juice and Orange Pulpwash

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Adulterations of citrus juices have evolved from simple dilutions with water, sugar and acids to highly sophisticated methods employing "tailor made" adulterants designed specifically to conceal adulteration. Orange pulpwash (water extraction of soluble fruit solids from orange pulp also referred to as WESOS or WEOS) as a substitution for or an adulterant of frozen concentrated orange juice (FCOJ) and single-strength orange juice (SSOJ) has become of great concern to the citrus industry and regulatory agencies, and an economic fraud on the consumer. Department of Citrus Rule 20-64.07 (3) states FCOJ packed in Florida and sold, shipped, or offered for sale or shipment in retail or institutional size containers shall not contain soluble solids recovered by aqueous extraction or washing of fruit pulp; in addition Rule 20-69.02 (1)(d) states that an imported product to be used in the production of FCOJ in Florida cannot contain soluble solids recovered by aqueous extraction or washing of fruit pulp (1). However, Federal Standard of Identity 146.146, for FCOJ, states "In its preparation, seeds (except embryonic seeds and small fragments of seeds that cannot be separated by good manufacturing practice) and excess pulp are removed, and a properly prepared water extract of the excess pulp so removed may be added" (2). The statement does not imply that a producer or packer may separately purchase orange pulpwash and add it to FCOJ or orange juice from concentrate. In fact an official opinion (Feb. 4, 1980) from Joseph P. Hile, Associate Commissioner for Regulatory Affairs for the Food and Drug Administration, to the State of Florida Department of Citrus states "It is clear, both from the plain words of the standard and the background, that only the "pulpwash solids" (a properly prepared water extract) of the excess pulp removed from the particular batch of oranges used in preparing the frozen concentrated orange juice may be added back to that juice and that pulp from other sources cannot be used. The actual amount of added pulpwash solids would, of course, vary somewhat with the particular batch of orange juice being processed." The consensus of industry and professional personnel involved is that the product de-

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scribed by Federal regulation 146.146 would contain an average of 6% and at most 10% pulpwash. Therefore, anything greater than that amount should be considered an adulterated product.

To reveal the extent of the adulteration problem, three national surveys were conducted by the Florida Department of Citrus from June 1979 through February 1980. The samples (FCOJ and SSOJ packed outside of Florida) were obtained from the retail market by Department of Citrus field personnel and shipped to our laboratory for analyses. In the second survey, 26 of 55 FCOJ samples tested were identified after analysis from container codes as packed in Florida--none had revealed any type of adulteration. Of the remaining 29 FCOJ samples, 86 percent indicated pulpwash adulteration in amounts up to 60 percent. Twenty-five percent (6 of 25) of the SSOJ samples indicated pulpwash adulteration in amounts to 40 percent. State of Florida Citrus Fruit Laws (3) govern the inspection, grading, certification, sale and shipment within or without the State of all citrus fruit and the products thereof whether canned or concentrated. The laws also govern the licensing or certification of citrus fruit inspectors by the United States Department of Agriculture. There are no required inspections, grading or certification of a product repacked by other distributors out of the state of Florida.

Scientists (4,5,6,7) at the University of Florida Agricultural Research and Education Center, Lake Alfred, Fla. conducted a comparison of aqueous extracts of orange pulp and reconstituted orange juice concentrates. Results showed that the chemical and physical characteristics of water extracts of orange pulp varied with pulp washing procedures and variety of fruit used. Also, flavonoids and water-soluble pectin were found in greater quantities in pulpwash than in their corresponding orange juices. Sawyer (8) conducted an investigation of the chemical composition of pulp extracts, and fresh and concentrated orange juices. He concluded from results of chemical analyses that a general composition of a juice may be determined, including type of adulteration.

Lifshitz et al. (9) investigated the purity of Israeli citrus juice by a multivariate method. They selected 5 analytical parameters and concluded that adulteration or dilution of 15% or more was very likely to be detected at a 1% level of significance. However, adulteration with orange pulpwash was not included in their study.

Schatzki and Vandercook (10) measured the chemical composition (total sugars, reactive phenols, total amino acids, arginine and γ -aminobutyric acid) of concentrated orange juice for manufacture from Arizona, California, Florida and Mexico and orange pulpwash samples from Florida. They investigated the use of non-parametric nearest neighbor classification techniques for the detection of adulteration with sugar, reducing sugars and citric acid. It was concluded that "sugared" pulpwash was often classified as pure concentrated orange juice for manufacture.

Vandercook and Rolle (11) investigated the ultraviolet absorption characteristics of alcoholic solutions of California-Arizona lemon juice. They reported that the ratio of the absorbance of 273-277 nm to the absorbance of 326-332 nm was essentially constant. From analyses of the spectra obtained of other fruit juices, they indicated if one were added to lemon juice its presence could be detected by a displacement of this ratio. Vandercook et al. (12) observed a significant increase in total polyphenolic absorbance at 330 nm, of lemon juice, with extraction pressure. However, there was no significant change in the A/B absorbance ratio (273/326).

Petrus and Dougherty (13) investigated the combined visible and ultraviolet absorption characteristics of alcoholic solutions of Florida Hamlin, Pineapple and Valencia orange juices. Shoulders or peaks were observed at 465, 443 and 425 nm of the visible spectrum (due mainly to the carotenoids present) and 325, 280 and 245 nm of the ultraviolet spectrum (due mainly to polyphenols, flavonoids and ascorbic acid, respectively). Absorption of diluted samples obeyed Beer's law over the wavelength range of interest. However, the 443/325 nm and 280/325 nm absorbance ratios remained essentially the same as before dilution. Ultraviolet absorption increased and 443/325 nm absorbance ratio decreased with increasing fruit extractor pressure while only slight changes were observed in the visible absorption. Spectra of alcoholic solutions of the rag and albedo components of the orange varieties showed very little visible but very strong ultraviolet absorbance. Whereas, spectra obtained of juice from juice sacs revealed it as the major contributor to the visible absorption, and having also strong ultraviolet absorption. Petrus and Dougherty (14) also investigated the spectral characteristics of mid- and late season frozen orange concentrates and their corresponding frozen orange pulpwash concentrates obtained from commercial processors. They concluded that alcoholic solutions of orange pulpwash samples were characterized by weak visible and strong ultraviolet absorption. They hypothesized weak visible absorption was due to the concentration of parent orange juice present, while the strong ultraviolet absorption was the result of parent orange juice present plus incorporation of rag and pulp and their water-extracted soluble solids into the product during the pulp washing process. Parent orange juice concentration present in the pulpwash product was estimated from the pulpwash to orange juice absorbance ratio at 443 nm.

The purpose of this presentation is to discuss the visible and ultraviolet absorption and room temperature fluorescence characteristics of alcoholic solutions of Florida produced orange juice and pulpwash samples, and to relate the characteristics to qualitative detection and quantitative approximation of adulteration of frozen concentrated and single-strength orange juices. Experimental details of our procedures may be found elsewhere (15).

Typical visible and ultraviolet absorption spectra obtained for alcoholic solutions of Florida Valencia orange juices are shown in Fig. 1 (please refer to references (13, 14, in press 15), for discussion of absorption wavelengths). Pineapple orange may be distinguished from the Valencia orange variety by generally stronger absorbance at 245 nm (ascorbic acid being the main contributor at this wavelength) and slightly lower overall visible absorbance. Visible absorbance spectra obtained from the Hamlin variety were slightly less than Pineapple, however, ultraviolet absorbance spectra were similar. To simplify the presentation only the above three varieties will be discussed (the effect of variety, maturity, extractor type and setting were investigated during the 1971-72 (13), 1972-73, and 1973-74 seasons, unpublished data).

Fig. 2 shows typical fluorescence excitation and emission spectra obtained from alcoholic solutions of Florida Valencia SS0J's. Fluorescence spectra obtained from Pineapple and Hamlin orange juices were similar to but of lower intensity than Valencia. Early in the Pineapple season the emission spectrum may appear as a flat apex (from about 310 to 333 nm). Early season Hamlin juice may produce an emission spectrum with 310 nm as the maximum dropping to an inflection at 333 nm. As the fruit matured 333 nm became the emission maximum. Maximum excitation and emission (for the three varieties) were also evident at 290-93 and 343 nm, respectively, producing excitation spectra similar to Fig. 2 (shoulder at 283 nm and slight inflection changes at 270 and 302 nm). Valencia variety exhibited greater fluorescence than Hamlin or Pineapple varieties.

A spectral (visible and ultraviolet) absorption curve comparison of reconstituted, commercial Florida produced, FC0J and orange pulpwash is shown in Fig. 3. The absorption shoulders and maxima were as previously discussed. It is evident that orange pulpwash had a much weaker unresolved visible absorption and very much stronger, resolved (at 280 nm) ultraviolet absorption than orange juice. It is also evident that the absorbance ratio at 443/325 nm would be lower and the sum of absorbance (at 443, 325 and 280 nm) would be greater for pulpwash than for orange juice. Water extraction of orange pulp and rag (during the pulp washing and finishing process) would tend to incorporate more rag and pulp and their water-extractable soluble solids into the product, thus increasing the ultraviolet and decreasing the visible absorption (13,14). Fig. 4 shows the comparison of the fluorescent excitation and emission curves obtained from solutions of orange juice and pulpwash. Maxima for orange juice appeared at 290 nm excitation and 343 nm emission with excitation shoulders or inflections at 270, 283, and 302 nm. Pulpwash revealed maxima at 270 and 302 nm with shoulders at 283 and 290 nm excitation and 353-55 nm emission. Pulpwash samples (not shown by Fig. 4) have also shown strong excitation maxima at 270 and 302 nm with a minimum at 280-85 nm. Alcoholic solutions of orange components (rag,

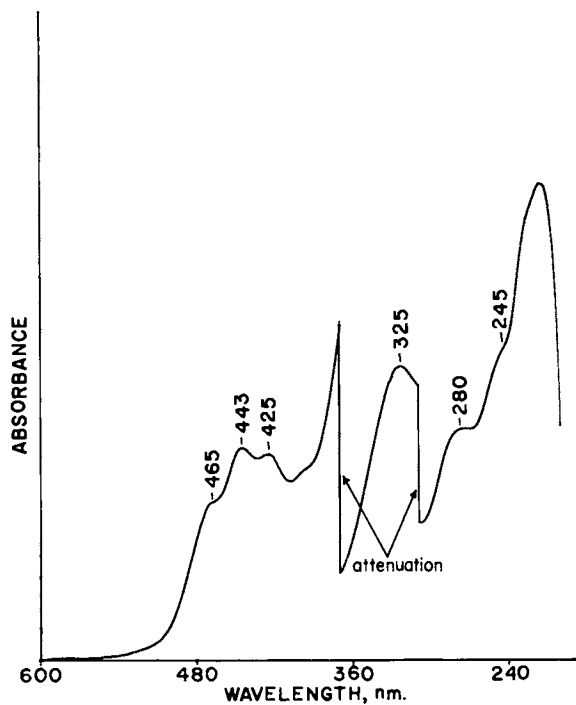


Figure 1. Visible and UV absorption spectra obtained from an alcoholic solution of Valencia orange juice

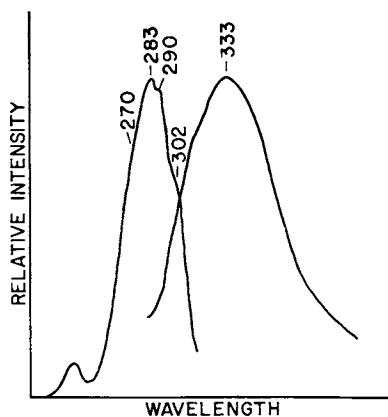


Figure 2. Fluorescence excitation (283 nm maximum) and emission (333 nm maximum) spectra obtained from Valencia orange juice

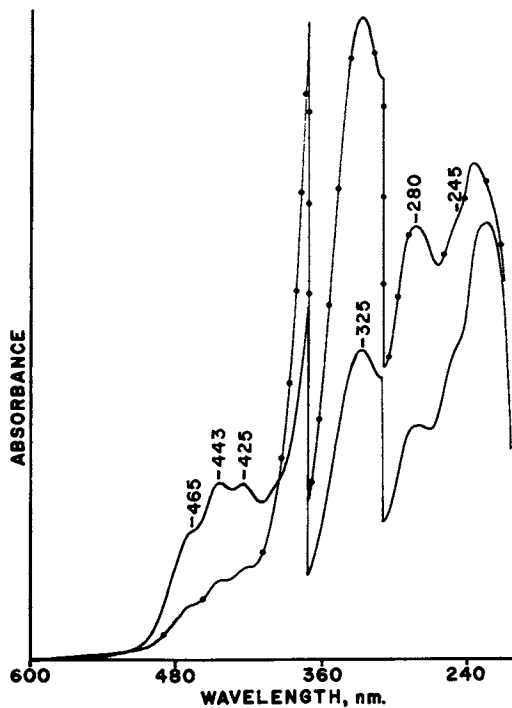


Figure 3. Spectral (VIS/UV) absorption curve comparison of commercial Florida-produced frozen concentrated orange juice (—) and orange pulpwash (●—●)

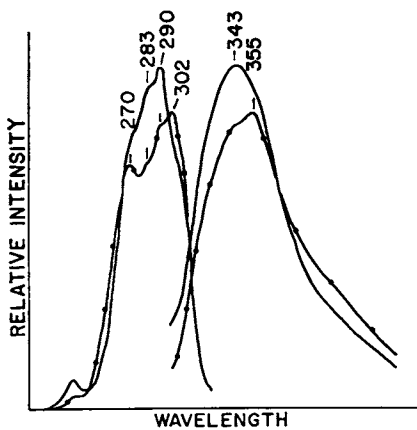


Figure 4. Comparison of fluorescence excitation and emission spectra of orange juice (—) and orange pulpwash (●—●)

albedo, juice from juice sacs) produced fluorescence spectra similar to FCOJ or SSOJ by the juice component, while the rag and albedo components produced spectra similar to pulpwash. Again pulp washing and finishing would tend to incorporate more rag and pulp and their water-extractable soluble solids into the product, thus an orange juice sample exhibiting fluorescence excitation peaks or pronounced shoulders at 270 and 302 nm would indicate the presence or use of pulpwash. Table I lists the excitation and emission wavelengths utilized for sample characterization. If an emission wavelength was absent for a sample under investigation the procedure was to set the emission monochromator at the desired wavelength and scan the excitation.

Table I. Fluorescence Excitation and Emission Characterization of Orange Juice and Pulpwash.

Excitation (nm)	Emission (nm)	Range Setting	Excitation Spectra	
			Orange Juice	Pulpwash
340	423	0.1	Strong	Much stronger
230	310	0.03	Weak	Weaker
283	333	0.1	Strong Maximum	Minimum
290	343	0.1	" "	Minimum or Inflection
302	353	0.1	Inflection	Strong Maximum
270	333	0.1	Inflection	Shoulder or Maximum

Fig. 5 and 6 show the absorbance sum and ratio distribution (normal curve constructed) of Florida commercial packed FCOJ reconstituted to 12.8° Brix. Sampling period was from 1972 through 1979. Absorbance sum of 416 samples showed a minimum of 2.002, maximum of 2.992, mean and standard deviation of 2.410 ± 0.164 , and coefficient of variation of 6.8%. Absorbance ratio revealed a minimum of 0.092 , maximum of 0.227, mean and standard deviation of 0.144 ± 0.026 , and coefficient of variation of 18%.

Fig. 7 and 8 show the absorbance sum and ratio distribution of Florida commercial packed frozen orange pulpwash concentrate (reconstituted or calculated to 12.8° Brix). Sampling period was from 1971 through 1979. Absorbance sum of 206 samples showed a minimum of 2.617, maximum of 4.992, mean and standard deviation of 3.781 ± 0.473 , and coefficient of variation of 12.5%. Absorbance ratio showed a minimum of 0.017, maximum of 0.112, mean and standard deviation of 0.048 ± 0.020 , and coefficient of variation of 41.7%.

Inspection of the distribution plots and data revealed more variation in pulpwash than FCOJ, indicating standardization of Florida FCOJ products. However, there are many processing variables in orange pulp washing, such as number of, and mechanical

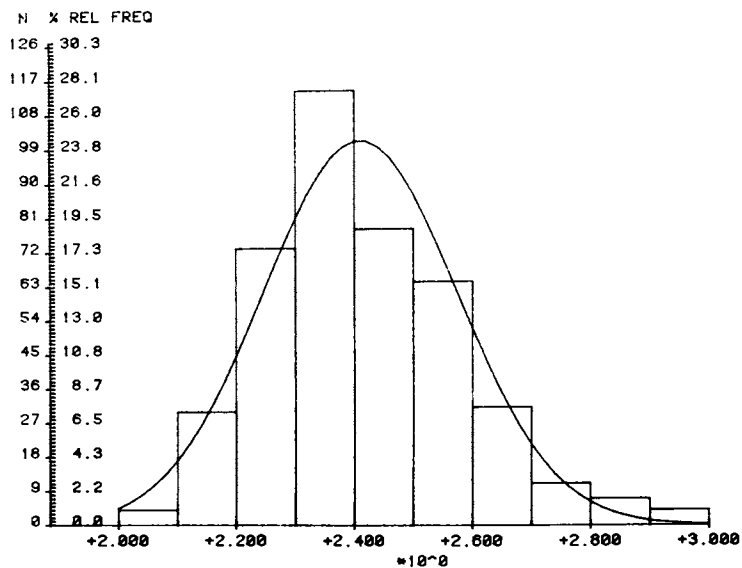


Figure 5. Distribution of absorbance sum (at 443, 325, and 280 nm) of Florida-packed FCOJ (N = 416; mean = 2.410; standard deviation = 0.164)

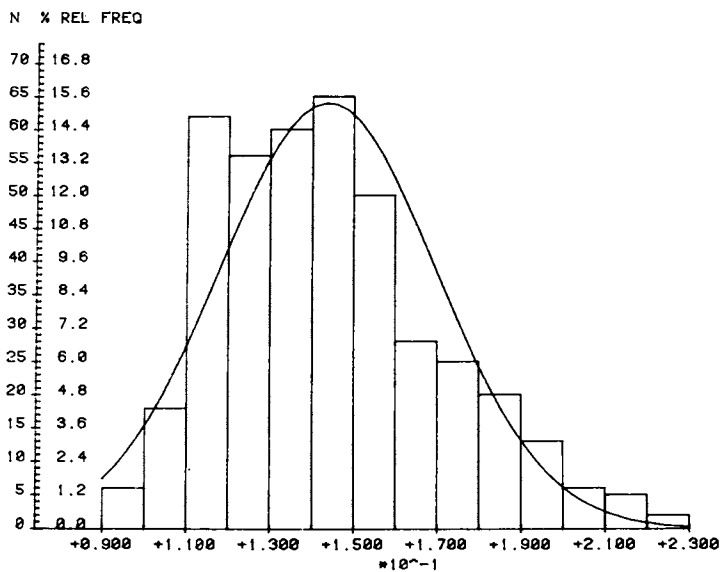


Figure 6. Distribution of absorbance ratio (at 443/325 nm) of Florida-packed FCOJ (N = 416; mean = 0.144; standard deviation = 0.026)

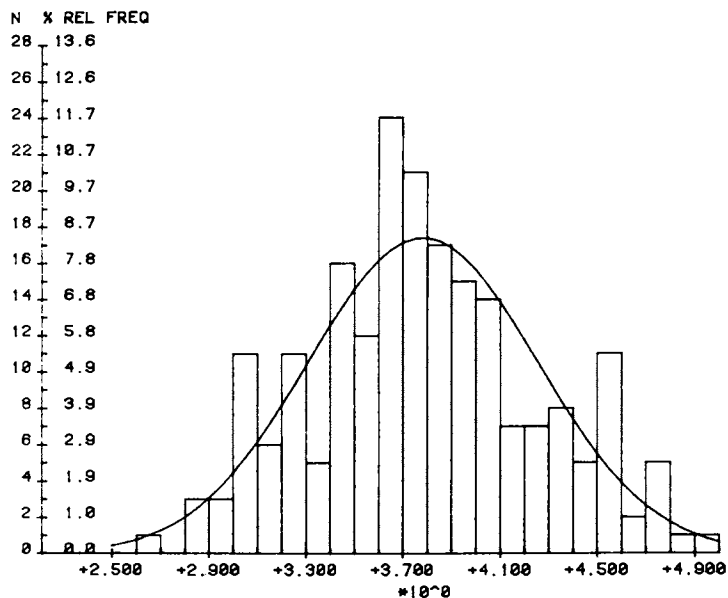


Figure 7. Distribution of absorbance sum of Florida-produced orange pulpwash (N = 206; mean = 3.781; standard deviation = 0.473)

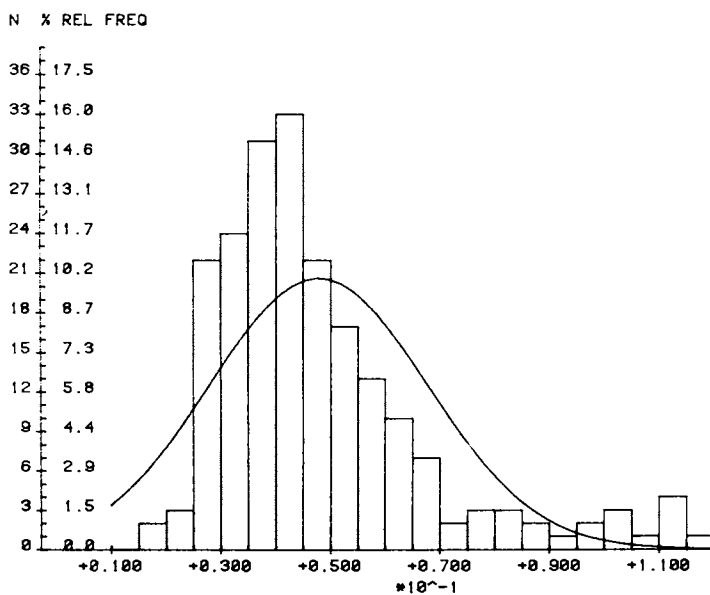


Figure 8. Distribution of absorbance ratio of Florida-produced orange pulpwash (N = 206; mean = 0.048; standard deviation = 0.020)

setting of finishers, and incorporation of fruit core and centrifuge discharge into the pulp washing process resulting in products of differing spectral characteristics. A primary or single stage finisher produced pulpwash may resemble the excitation curve shown in Fig. 4, whereas pulpwash obtained from a multi-stage finisher may show strong excitation at 270 and 302 nm with a minimum at 280-85 nm.

The distribution plots also reveal a slight overlapping region. There were four FCOJ samples (1.0% of total) having an absorbance sum greater than 2.900 and seven pulpwash samples (3.3%) having a sum less than 3.000. Distribution of absorbance ratio revealed more overlap than absorbance sum. There were 24 FCOJ samples (5.8%) having an absorbance ratio less than 0.110 whereas pulpwash had 12 greater than 0.090 (5.8%). There appeared to be good separation of the general populations.

Model systems composed of mixtures of reconstituted commercially produced FCOJ and orange pulpwash were prepared, and their alcoholic solutions investigated for characteristic visible and ultraviolet absorption, and room temperature fluorescence. Four models using 3 different FCOJ's and 4 different pulp washes were investigated from 100 percent orange juice through 100 percent pulpwash. Statistical analysis of the data, (with absorbance sums at 443, 325 and 280 nm, absorbance ratios at 443/325 nm and fluorescence ratios at 302/284 nm) in all cases showed each model system producing a coefficient of determination $r^2 > 0.99$. Multiple linear regression analysis of each system utilizing the absorbance sum and ratio produced coefficients of determination $R^2 > 0.999$. Multiple regression analysis of the combined data obtained from the 4 models produced the equation:

$$y = 241.768 - 46.776(\Sigma) + 19.043(\ln \frac{443}{325} \text{ nm})$$

where y equals approximate percent orange juice, and $100-y$ equals approximate percent pulpwash. This resulted in a high coefficient of determination $R^2 = 0.904$, indicating 90.4% of the variation in orange juice content may be explained by the absorbance sum and natural log of the ratio. The results approximated the orange juice content for systems of orange juice adulterated with pulpwash, however, it failed when the adulteration was further complicated by dilution with water. Since adulteration of orange juice with pulpwash increased the absorbance sum (linear function) it was apparent that further adulteration by dilution with water would decrease the sum (linear function). The adulterated sample could then indicate an absorbance sum in the range found for orange juice leading to erroneous predictions of orange juice and pulpwash contents by multiple regression equations. Since simple dilutions of orange juice resulted in essentially no change in the absorbance ratio, and addition of pulpwash to orange juice resulted in an absorbance ratio decrease (natural log function), it appeared likely a mathematical system could be established relating the sum and ratio to orange juice and/or pulpwash contents and/or dilution.

The following equations were derived using the following constants:

$$Z = Av \sum_{pw} - Av \sum_{oj} = 3.484 - 2.410 = 1.074 \quad 1$$

where $Av \sum_{pw}$ and $Av \sum_{oj}$ were average absorbance sums for pulp-wash (at 11.8° Brix) and orange juice (at 12.8° Brix) respectively.

$$y = \ln Av R_{oj} - \ln Av R_{pw} = \ln 0.144 - \ln 0.048 = 1.099 \quad 2$$

where $\ln Av R_{oj}$ and $\ln Av R_{pw}$ were natural log of average absorbance ratios for orange juice and pulp wash respectively.

Then

$$x = \ln R_{unk} - \ln Av R_{pw} = \ln R_{unk} - \ln 0.048 \quad 3$$

where $\ln R_{unk}$ was the natural log absorbance ratio of the unknown sample.

The average absorbance sum correction factor

$$a = \frac{x}{y} = \frac{x}{1.099} \quad 4$$

and absorbance correction value

$$b = Z(a) = 1.074(a) \quad 5$$

and the corrected average absorbance sum

$$c = Av \sum_{pw} - b = 3.484 - b \quad 6$$

At this point it may be explained that if $x = 0$ (when $\ln R_{unk} = \ln Av R_{pw}$) then $a = 0$, therefore $b = 0$ and the corrected absorbance sum $c = Av \sum_{pw} - 0 = 3.484$ and the product would contain pulp wash in pure or diluted form.

However, if $x = 1.099$ (when $\ln R_{unk} = \ln R_{oj}$) then $a = 1$, therefore $b = 1.074$ and the corrected absorbance sum $c = 3.484 - 1.074 = 2.410$ which is equal to the average absorbance sum for orange juice and the product would indicate orange juice in pure or diluted form.

$$\% TCM = \frac{\sum_{unk}}{c} \times 100 \quad 7$$

where % TCM is the approximation of total citrus material present in the unknown sample and \sum_{unk} is the unknown sample absorbance sum.

Then

$$\% \text{ TCM(a)} = \% \text{ OJ} \quad 8$$

and

$$\% \text{ TCM} - \% \text{ OJ} = \% \text{ PW} \quad 9$$

and

$$100\% - \% \text{ TCM} = \% \text{ Dilution} \quad 10$$

The following cases illustrate use of the above equations:

Case 1. If the results indicate a combination of orange juice, pulpwash and dilution, then equation 8, 9 and 10 are reported as calculated and product formulation equals 100 percent.

Case 2. If results indicate only orange juice and pulpwash, then results of equations 8 and 9 are set proportional to 100% as % OJ: % TCM = x: 100 and % PW: % TCM = x: 100 respectively. Solved for x, product formulation equals 100 percent.

Case 3. If results indicate orange juice, or orange juice with dilution, then equations 7 and 10 are reported as calculated and product formulation equals 100 percent.

Case 4. If results indicate only pulpwash or pulpwash with dilution, then equations 7 and 10 are reported as calculated and product formulation equals 100 percent.

The equations were performed on the data obtained from the 416 FCOJ samples previously discussed. Qualitatively the absorption and fluorescence spectra did not indicate the presence of orange pulpwash. Therefore, the juices were examples of Case 3, and orange juice content reported as % TCM (equation 7). Statistical analysis of the results showed a mean of 99.6% TCM and standard deviation of 8.5.

To further verify the equations, 29 unknown samples (4 sets) composed of various combinations of orange juices, and/or pulp-washes and/or product dilutions were prepared by others for analysis. Calculated values were correlated with actual values and gave the following coefficients of determination: orange juice content $r^2 = 0.942$; pulpwash content $r^2 = 0.981$; and dilution $r^2 = 0.937$. Even with significant coefficients of determination, it must be stressed that the equations provide only approximations of sample formulations. The constants used in the equations were based on results obtained from commercial Florida

citrus products and could vary with other citrus producing regions.

The following examples were selected from the aforementioned national surveys conducted by the Florida Department of Citrus. The examples were packed outside Florida and obtained from the retail market. They will serve to illustrate both qualitatively and semi-quantitatively the information which may be obtained from the spectra and equations.

Visible and ultraviolet absorption and fluorescence spectra, obtained from alcoholic solutions of a commercially packed (out of the State of Florida) FCOJ, are presented in Fig. 9, 10, 11 and 12. Qualitatively, Fig. 9 reveals a lack of resolution in the visible absorption region and a well resolved peak at 280 nm. Comparison with Fig. 1 and 3 shows its absorption characteristics to be more similar to those of orange pulpwash in Fig. 3, indicating adulteration by pulpwash addition. Fluorescence excitation spectra (Fig. 10, 11) reveal well defined peaks at 270-75 nm, and Fig. 12 a shoulder at 270-75 nm. The spectra appear deformed when compared to Fig. 2 and 4 of pure orange juice. However, characteristics are similar to those obtained from pulpwash and very similar to those obtained from prepared model systems. Qualitatively both visible and ultraviolet absorption, and room temperature fluorescence indicate the presence of pulpwash in the FCOJ sample. The spectra are complementary. Absorption also did not indicate further adulteration by dilution which would have been denoted by weaker overall absorption and a shift at 227 nm to shorter wavelength. The sum of absorption at 443, 325 and 280 nm is $0.098 + 1.040 + 1.622 = 2.760$ absorbance units. Florida State statute 20-64.07(1)(a) requires FCOJ to be 44.8° Brix which reconstituted to 12.8° Brix (16) and Federal standards, Section 52.2582(a), require 41.8° Brix, reconstituted to not less than 11.8° Brix (17). Therefore, the sum of absorbance is multiplied by the ratio of 12.8 to 11.8° Brix, with a corrected sum of 2.995. The sample absorbance ratio at 443/325 nm is $0.098/1.040$ which is equal to 0.094. If the sum and natural log ratio values are substituted into the regression equation:

$$y = 241.768 - 46.776(\Sigma) + 19.043(\ln \frac{443}{325} \text{ nm})$$

the sample was found to contain approximately 57% orange juice and 43% pulpwash ($100-57 = 43$). If the sum and natural log ratio are used with equations 1 through 10, the sample was found to contain approximately 65% orange juice and 41% pulpwash (106% TCM) and there was no indication of further dilution. The sample consisted of only orange juice and pulpwash, and is a Case 2 example. Therefore, solving the proportions ($65:106 = x:100$ and $41:106 = x:100$) resulted in a product formulation of approximately 61% orange juice and 39% pulpwash. The values are comparable to those obtained using the regression equation.

Visible and ultraviolet absorption and room temperature fluorescence excitation and emission spectra obtained from alcoholic

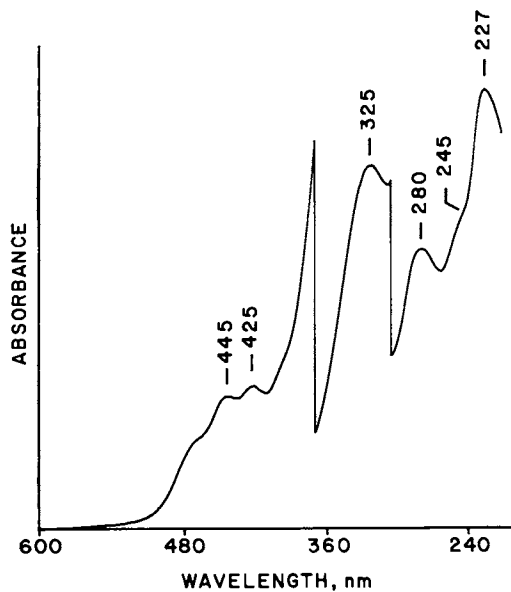


Figure 9. Visible and UV absorption spectra obtained from a commercially packed (outside Florida) FCOJ

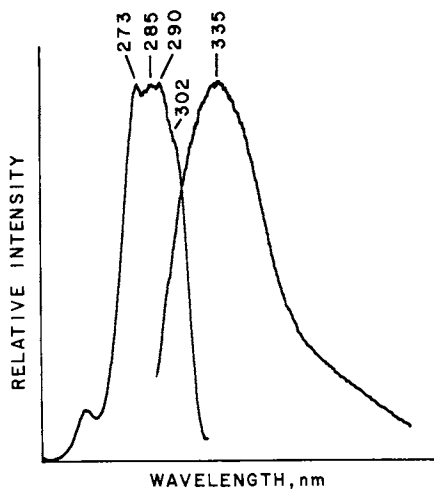


Figure 10. Fluorescence excitation (285 nm) and emission (335 nm) spectra obtained from a commercially packed (outside Florida) FCOJ

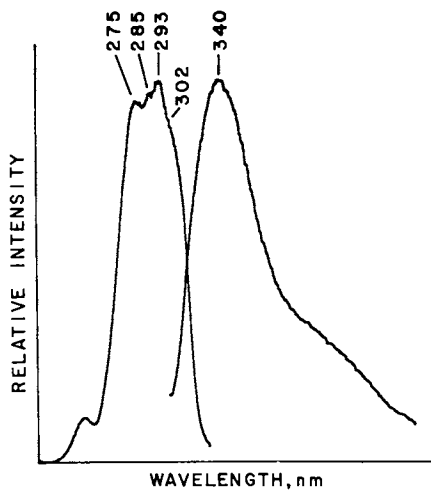


Figure 11. Fluorescence excitation (293 nm) and emission (340 nm) spectra obtained from a commercially packed (outside Florida) FCOJ

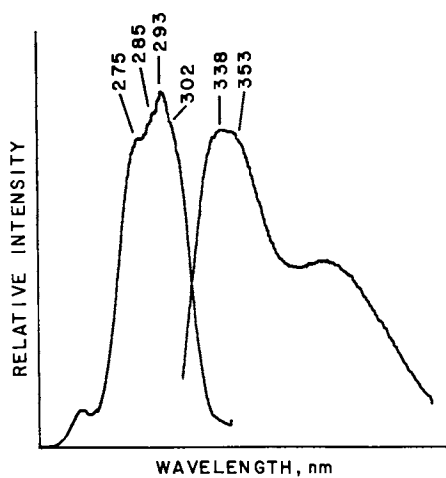


Figure 12. Fluorescence excitation (302 nm) and emission (353 nm) spectra obtained from a commercially packed (outside Florida) FCOJ

solutions of a single-strength orange juice are presented in Fig. 13, 14, 15 and 16. The sample was of commercial origin packed out of the State of Florida and labeled 100% orange juice from concentrate, unsweetened, and no sugar added. Fig. 13 reveals a lack of resolution in the visible absorption region and well-resolved peak at 280 nm which is typical of pulpwash addition. Weak overall absorption and the peak shift to shorter wavelength at 211 nm indicate dilution. Fluorescence excitation (Fig. 14, 15 and 16) all indicate some deformity when compared to Fig. 2, and is typical of pulpwash addition. Absorption and fluorescence spectra must be complementary. Because of the apparent dilution involved, estimation of the orange juice and pulpwash contents cannot be accomplished using the regression equation. The absorbance sum ($0.056 + 0.510 + 0.850$) corrected for 12.8° Brix was 1.536 absorbance units and the absorbance ratio was 0.110. The low absorbance sum is well outside the distribution curve for orange juice and indicates product dilution. The absorption ratio is within two standard deviations of the orange juice distribution and just outside three standard deviations of the pulpwash distribution in the overlap area. The results indicated orange juice, pulpwash and dilution, therefore, the sample is a Case 1 example. Calculations using equations 1 through 10 provided the following approximate formulation: 43% orange juice, 14% pulpwash and 43% dilution. It must be pointed out that the formulation contained 14% pulpwash and the total citrus material (TCM) calculated was 57%. Therefore, pulpwash comprised 25% of the total citrus material. Sensory evaluations (unpublished data, P. J. Fellers, Fla. Dept. of Citrus, 1979) of the sample tended to substantiate the analysis. The evaluations revealed a slightly bitter taste almost totally lacking in fullness of normal orange juice flavor, and somewhat like isotonic orange beverage (a thirst-quencher type beverage developed at the University of Florida Agricultural Research and Education Center, Lake Alfred, Fla. having 25% orange juice solids and very small amounts of certain salts). The flavor grade was a very unsatisfactory "dislike moderately" on a 9-point hedonic scale. Bitterness can be indicative of pulpwash addition and blandness or lack of flavor can be indicative of dilution. Sugar analysis (unpublished data, S. V. Ting, Fla. Dept. of Citrus, 1979) of the sample revealed about 76% sucrose as percent total sugars, whereas the average for single-strength orange juice was about 50%. This also was supportive evidence indicating dilution of the sample.

In conclusion, it has been shown that the visible and ultra-violet absorption, and room temperature fluorescence spectra, obtained from alcoholic solutions of orange juice and related products, may be used for product characterization. The complementary absorption and fluorescence spectra may be utilized for the qualitative detection of adulteration of reconstituted frozen concentrated and single-strength orange juice with pulpwash. Previous investigations (chemical and elemental profile analyses)

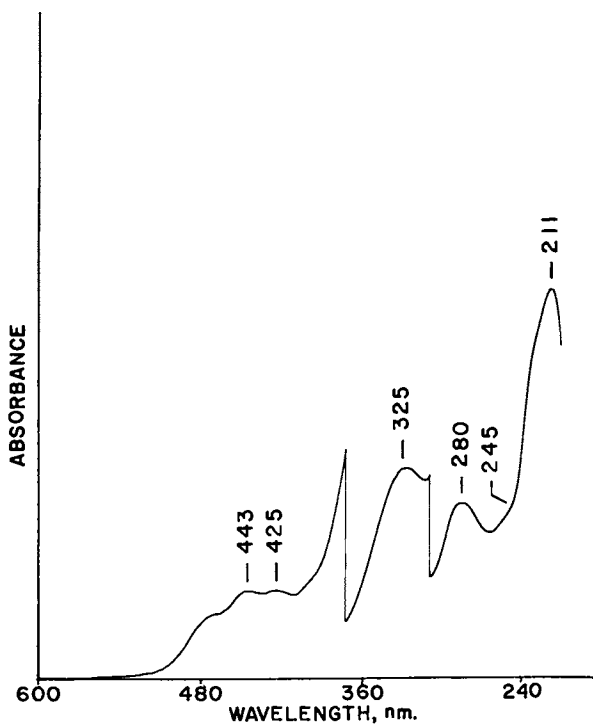


Figure 13. Visible and UV absorption spectra obtained from a commercially packed (outside Florida) SSOJ

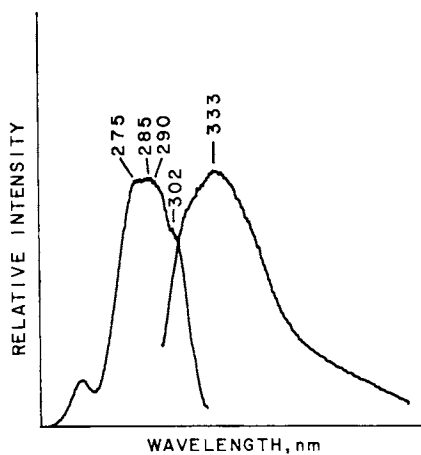


Figure 14. Fluorescence excitation (285 nm) and emission (333 nm) spectra obtained from a commercially packed SSOJ

Figure 15. Fluorescence excitation (290 nm) and emission (340 nm) spectra obtained from a commercially packed SSOJ

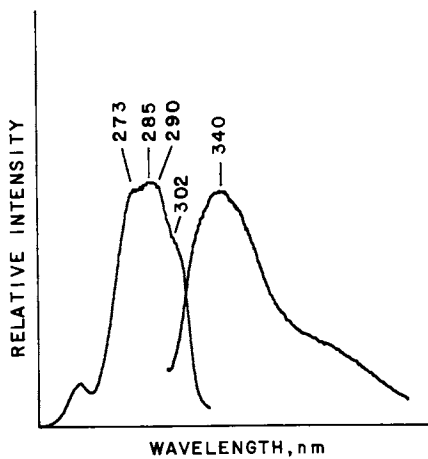
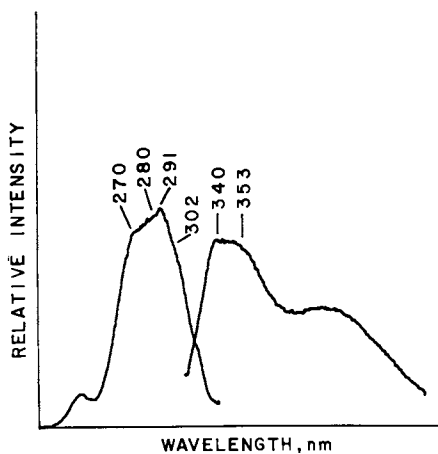


Figure 16. Fluorescence excitation (302 nm) and emission (353 nm) spectra obtained from a commercially packed SSOJ



have been concerned mainly with dilutions of the product followed by fortification with certain inexpensive chemicals or elements to disguise the adulteration. However, simple dilution of orange juices adulterated with orange pulpwash would result in chemical and elemental profiles similar to orange juice and thus confuse these types of analyses.

Multiple regression analyses of the absorbance data produced an equation (utilizing the absorbance sum and ratio) for the estimation of orange juice and pulpwash present in a product. The equation worked well for orange juices adulterated with pulpwash that were not diluted. Therefore, a series of equations was derived, using model systems and absorbance data, to approximate orange juice and/or pulpwash content and/or dilution of an orange juice sample. The constants used were obtained from analyses of commercial Florida citrus products and may vary with other citrus producing regions.

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