SEASONAL CHANGES IN [redacted] ORANGES [redacted] AND THE EFFECT OF CANNING AND STORAGE ON THE CONSTITUENTS OF THE JUICE

A Thesis submitted for the Degree of
Doctor of Philosophy
in the University of London

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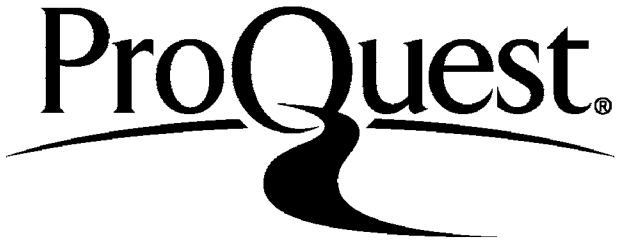
Royal Holloway College 1984



ABSTRACT

Seasonal changes in the Persian orange (*Citrus sinensis* var. Sia-Varaz) and the effect of canning and storage on the juice were studied. The major factors under consideration were the physical characteristics: weight, size, volume of juice, colour of peel; and chemical characteristics: free sugars, vitamin C, free amino acids and lipid content.

In order to determine the optimum state of maturity of the orange, samples were harvested at regular monthly intervals and analysed.

1. Throughout fruit development there was a continuous increase of juice, fruit size, and total soluble solids while total acidity decreased. These parameters were sufficiently different to enable the separation of the development cycle into three distinct phases: Cell division, Cell enlargement and Maturation of the fruit.

   Colour break from yellow to orange occurred between December and January but the oranges were assessed to be mature for harvesting by February and March.

2. Five sugars were identified: xylose, galactose, fructose, glucose and sucrose. Xylose soon disappeared while galactose was only found in minor quantities. Sucrose and reducing sugar content increased with fruit development. In the mature orange the ratio of sucrose:glucose:fructose was very close to 2:1:1.

3. While vitamin C content per 100 g pulp showed no significant change over the period of study the amount per orange pulp increased approximately four-fold.

4. Seventeen amino acids were identified. Increase in proline and arginine during December and January may be used as an indication of the onset of maturation.

5. Changes in lipid constituents were noted but, in general, no definite pattern could be established.

The processing procedure involved heating the juice at 98°C for 30 seconds and then sealing in lacquered A1 cans. Analyses were carried out at two-monthly intervals.

6. Although total free sugars, vitamin C and colour of the juice were not affected by processing, significant increases in total free amino acids and lipids were noted.

7. Storage of canned juice at 7°C for twelve months showed no significant effect on total free sugars, vitamin C, free amino acids and flavour. However, total lipid was significantly decreased after eight months.

8. Storage of canned juice at 30°C brought about significant losses of total free sugars after twelve months, of vitamin C after two weeks, and of free amino acids after two months, and of total lipids after two months. The flavour of the juice, judged by a taste panel, had deteriorated significantly after one month's storage.

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SECTION I

SEASONAL CHANGES IN SIA-VARAZ ORANGES

Abbreviations used in the text

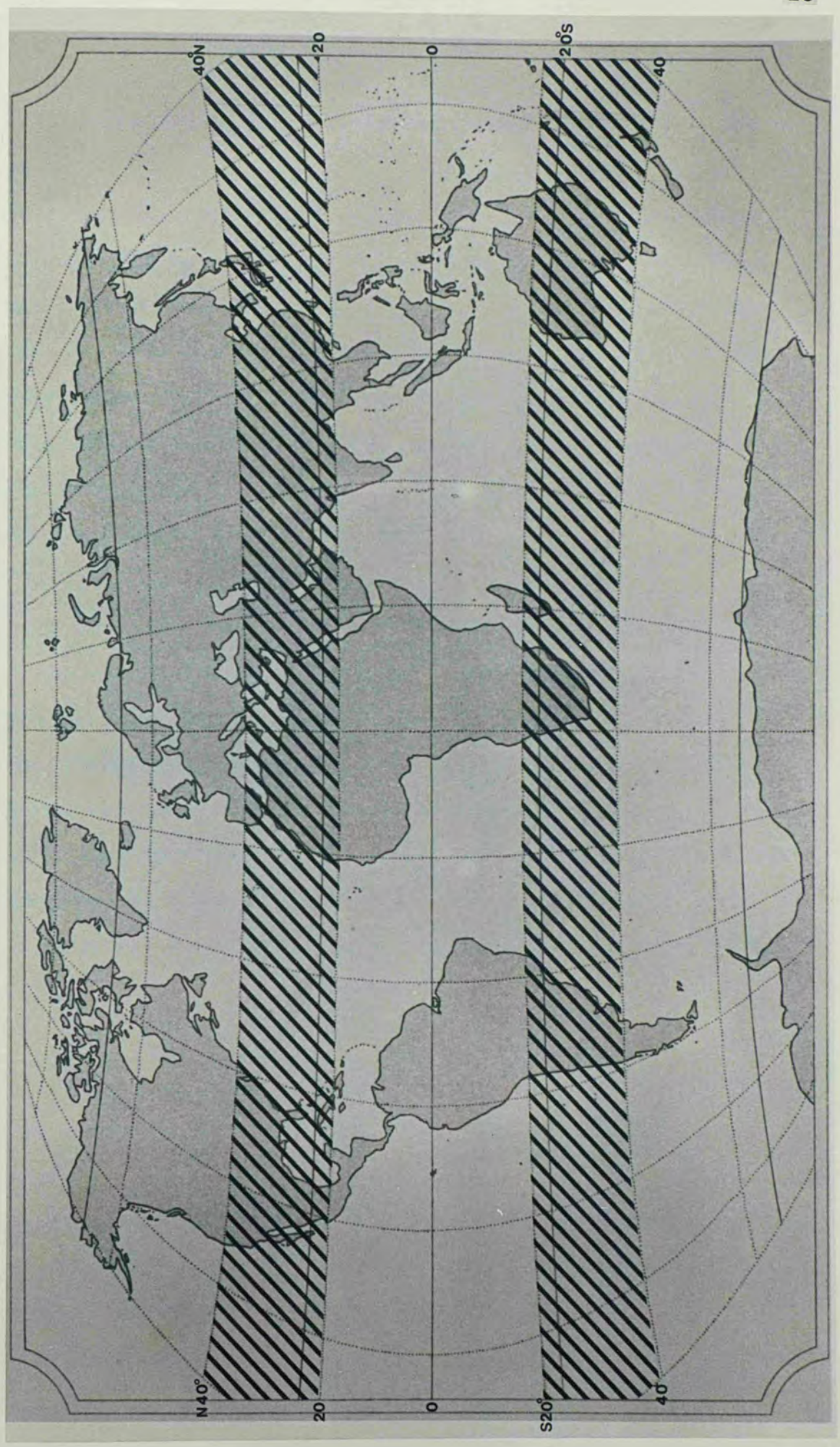
Ammonia	NH_3
Ceramide monohexosides	CMH
Digalactosyl diglycerides	DGDG
Diglycerides	DG
Free amino acids	FAA
Free fatty acids	FFA
Free sterols	FS
Fructose	Fru
Galactose	Gal
Glucose	Glu
Hydrocarbon	HC
Hydroxymethylfurfural	HMF
Lyso-phosphatidic choline	LPC
Monogalactosyl monoglycerides	MGMG
Monoglycerides	MG
Phosphatidic acid	PA
Phosphatidyl choline	PC
Phosphatidyl ethanolamine	PE
Phosphatidyl glycerol	PG
Phosphatidyl inositol	PI
Phosphatidyl serine	PS
Relative humidity	RH
Recommended dietary allowance	RDA
Single strength orange juice	SSOJ
Sterol glycoside	SG
Steryl esters	SE
Sucrose	Suc
Triglycerides	TG
Trimethylsilyl	TMS
Xylose	Xyl

GENERAL INTRODUCTION

The importance of citrus to agriculture and the world's economy is demonstrated by its wide distribution and large-scale production. Citrus is grown throughout the world in tropical and subtropical climates where there are suitable soils and sufficient moisture to sustain the trees and where it is not cold enough to kill them. The producing regions roughly occupy a belt spreading approximately 35°N and S of the equator (Fig. 1).

The main commercial areas are in the subtropical regions at latitudes higher than 20°N or S of the equator. The total planted area of the world's 49 citrus-producing countries is estimated at slightly over 1.6 million ha. Much of the world's citrus is grown for local use and a large number of trees are scattered or away from commercial transport. The Mediterranean area and North and Central America contain about 80% of the world's crop, while the remaining 20% is distributed in the Far East (10%), South America (6%) and in other Southern Hemisphere countries, including South Africa and Australia (4%). The Mediterranean area supplies about 80% of the total fresh fruit export and 8% of the processed products. North and Central America supply only about 9% of the fresh fruit export, but over 80% of the processed products; most of them being from the United States. Oranges constitute over 75% of the total production with lemons and grape-

Fig. 1. Citrus belts of the world in the subtropical regions of the northern and southern hemispheres (Cooper and Chapot, 1977)


40°N
20
0
20°S
40
N40°
20
0
S20°
40

fruit each accounting for approximately 10% (Burke, 1967).

The most important of all citrus fruit is the sweet orange (Citrus sinensis) which is widely grown and which has its own characteristic variety in each region of the world. Over a hundred varieties have been described to date (Hume, 1957), including the Washington Navel of California, the Parson Brown, Hamlin, and Pineapple of Florida; Shamouti of Israel; Cadenera of Spain; Para of Brazil; Ovale and Tarocco of Italy.

The introduction of the orange to Iran was as a result of visits by Portuguese ships to the southern parts of Iran in the Persian Gulf during the late 16th century. The orange plantations in Iran were started by using orange seeds and this was the common practice until 1934. After that, the use of seed plantation was replaced by grafting because the seedlings of these sweet oranges were very susceptible to Phytophthora (causing foot rot) and to the cold.

Although the citrus is now grown in both the northern and southern parts of Iran, the bulk of production is in a 2-km wide strip between the Caspian Sea and the Alborz Mountains known as the Caspian littoral constituting 22,000 hectares. The most commonly grown orange variety in the northern Iran is the Sia-Varaz which occupies 70% of the above area with an average yield of 15 tons per hectare. Its economical importance is reflected by the

fact that it provides £57,000,000 per annum to the local farmers and also provides temporary jobs for 800,000 people at harvesting time.

Most of the oranges used in the north are consumed in the fresh form, but to facilitate their transport to the southern regions, the fruit is also canned. At present there is only one orange juice processing factory in Iran and this has an output of 1,800 tons of canned Single-Strength Orange Juice (SSOJ) per annum. An improvement in canning procedures, coupled with increased production by building new factories, would help the country to fulfil its indigenous demand for oranges, some of which have to be imported at present.

To obtain the best results, the oranges must be fully mature at the time of harvesting, and it is desirable that the oranges are "tree ripened". The reason for this is that certain fruits (e.g. apples and pears) contain starchy reserves which are largely converted to sugar during ripening. In this case, they may be mature before they are harvested but are only edible sometime afterwards when they obtain their characteristic soft, juicy and aromatic qualities. Oranges are very different in this respect. They contain practically no starch (Braverman, 1933) and consequently do not undergo any change in composition after being detached from the tree. In other words, if an orange is not ripe at the time of harvest it never will be. Instead of increasing in quality as do

apples and pears, oranges lose quality. Therefore, orange fruits must be "tree-ripened" since they do not mature once they are harvested.

Most of the literature dealing with seasonal changes of oranges relates to maturity and efforts have been made to define this on the basis of their chemical composition. Some of the earliest studies on the compositional changes as a function of maturation involved total soluble solids, sugar contents, and total acid contents (Copeman, 1934; Stahl, 1935; Harding et al., 1940; Bartholomew and Sinclair, 1943; Roy, 1945). Much of this early literature has been reviewed by Rose et al. (1951) and by Sinclair (1961). Other studies in chemical constituents during sweet orange ripening were conducted on vitamin C (Harding et al., 1940; Eaks, 1964), pectic substances (Gaddum, 1934; Sinclair and Jolliffe, 1958, 1961; Rouse et al., 1964), organic acids (Clements, 1964; Rasmussen, 1964), total nitrogen and free amino acid content (Clements and Leland, 1962a, b; Ting, 1967; Vandercook and Price, 1972; Zamorani et al., 1972), lipids and their constituents (Nordby and Nagy, 1969; Nagy et al., 1978; Nordby and Nagy, 1977, 1979) carotenoids (Higby, 1963; Gross, 1977), and ethanol (Davis, 1970; Rasmussen, 1975).

A great deal is known about a number of orange varieties such as Washington Navel, Hamlin, Pineapple, Valencia, and Shamouti but not much about the others.

No concerted work has been carried out to date on the Sia-Varaz variety although it is commercially important in Iran. The orange juice is usually processed as single-strength orange juice (SSOJ) in Iran and this was carried out in the present study. The juice produced by the only factory in Iran is mostly consumed in the south of the country where the average spring, summer and autumn temperature is higher than 30°C. It was therefore important to study the effect of storage at 30°C on the physical and chemical constituents of the SSOJ. This was also studied here as was the effect of storage at the lower temperature of 7°C, a temperature at which the SSOJ is kept in the warehouses of the north.

The present study is divided into two main sections. The aim of the first section was to determine the optimum time for harvesting the oranges for eating fresh or for subsequent processing. In order to assess this, the analyses included studies of physical and chemical characteristics, sugars, vitamin C, amino acids and lipids. The second section was concerned solely with the effect of canning and canned storage at 7° and 30°C for 12 months on the same physical and chemical components studied in the first section.

CHAPTER I

CONDITION OF GROWTH AND CHARACTERISATION OF THE SIA-VARAZ ORANGE

INTRODUCTION

From fruit set to maturity, citrus fruits pass through three well-defined stages (Bain, 1958). Generally, the term 'immature' orange refers to the fruit within the first two stages of its growth, i.e. when the peel and flesh is pale green to green. Mature fruits (third stage) usually have yellow to orange coloured peel and are by then edible or desirable as a fruit to be eaten.

Stage 1, referred to as Rapid Cell Division, lasts between 4-9 weeks after fruit set. Increase in fruit size and weight during this stage is due mainly to growth of the peel by cell division. Juice sacs primordia also continue to form right up to the end of this stage.

Stage 2 is known as Cell Enlargement during which time the fruit increases in size due to cell enlargement. The juice vesicles expand, accompanied by an increase in juice content. The peel begins to change colour as the fruit approaches maturity.

In stage 3, or the Maturation Period, the colour of the peel passes from pale green through yellow and finally to orange. The acidity of the juice continues to decrease

while the juice vesicles continue to increase in size.

The development and maturation of oranges are influenced by the environmental and cultural factors under which the fruits are grown. These collectively include temperature, light, relative humidity (RH), rainfall, rootstock, soil texture, age of the tree and density of planting.

Temperature

Total available heat is probably the single most important factor in determining the growth rate and time of ripening of citrus fruits (Jones, 1961). The prevailing temperature of a growing area not only influences the internal quality of the fruit but also its external appearance and texture (Caprio and Harding, 1935; Jones et al., 1962 ; Cooper et al., 1963).

Light

The duration and intensity of light affect the quality at harvest. In a series of papers on the effect of light on the quality of Wahington Navel oranges, Sites and Reitz (1949, 1950) found that fruits exposed to the sun were lighter in weight, with thinner peel, higher in solids and lower in acids and in juice compared to those that were shaded or those inside a canopy. Exposed fruits are those which are exposed to sunlight, whereas shaded fruits are those from crowded trees and trees being heavy foliaged which prevents the sun from getting to the fruit.

Relative Humidity (RH)

High relative humidity has a positive effect on size, weight, acidity, vitamin C, and volume of the juice and a negative effect on peel thickness, soluble solids, solid/acid ratio, and colour of the peel in Washington Navel oranges (Cooper et al., 1963).

Rainfall

Supply of water to the plant should be adequate to ensure a product of high quality. When rainfall is insufficient during the growing season, irrigation is necessary. On the other hand, excessive rainfall has its disadvantages. Washington Navel oranges exposed to prolonged rainy weather develop water spot (Smoot et al., 1971). The underlying tissue, usually near the navel, swells where it absorbs water. In subsequent warm and dry weather, water soaked areas become dry, slightly sunken and brownish in colour. Sites and Camp (1955) have reported that the abundance of rainfall or excessive irrigation affects total soluble solids and acidity of the fruit.

Soil Texture

Soil moisture may be related to soil texture. Chandler (1965) stated that fruits ripen a little earlier on trees growing in a sandy or gravelly soil than in clayey soil. In a poorly drained soil, the soil inter-spaces are water-filled and aeration is thus reduced.

Rootstock

The effect of rootstock in citrus fruit quality has been well documented. Kefford and Chandler (1970) summarized this work and generalized that rough lemon rootstocks give high yield but with low juice, acidity, and total soluble solid contents. The reverse effects were observed in Trifoliate orange, Tango and Cleopatra stocks. Harding et al. (1940), working on different varieties of oranges, reported that oranges grown from seeds had higher values of vitamin C, total soluble solids, and juice compared to Valencia, Jaffa, Hamlin and Conner oranges grafted on rough lemon rootstock.

Density of Planting

Variations in the density of planting will affect the quality of fruits. The closer the planting, the less sweet will be the fruits. This is due to the fact that in close planting, the trees will get less sunlight, therefore the rate of photosynthesis will be slower, causing the synthesising of sugars in the plant to be slow (Sites and Reitz, 1949, 1950a).

Age of the Tree

Age of the tree may influence the size and quality of the fruit. Older orange trees were shown to bear small fruits with thin peel, but high in total soluble solids (Marloth and Basson, 1959). In general, small fruits tend to be sweeter than large ones.

Component Parts of the Intact Fruit

Since chemical analyses are often expressed as concentration of certain chemical constituents on the basis of the weight or percentage of a component part, a knowledge of the changes and characteristics of these component parts is necessary in a study of biochemical changes in developing fruits. The different parts of an orange fruit are called the flavedo, albedo, carpels, and seeds (Fig. 4). The flavedo is the outer coloured layer of the orange peel containing the chromoplasts and oil glands; the latter, located at different depths in the flavedo tissues, are the source of orange peel oil. The albedo is the inner white portion of the peel and is spongy in nature. The carpels or locules, better known as citrus segments or pulp, contain many juice vesicles or juice sacs. Within the juice sacs are many thin-walled juice cells (Davis, 1932). At maturity, the vacuoles of these juice cells are full of juice. When the membranes are broken, the juice, together with some thin cell walls and cell contents, is released. Commercially, the fibrous membranes separating the carpels and the central pithy core are termed the "rag". The seeds are the source of citrus seed oil.

On a whole-fruit basis, the percentage distribution of the component parts of the orange fruit differs according to its stage of development.

GENERAL MATERIALS AND METHODS

Sia-Varaz orange trees may start blooming in early May and continue until early June, depending largely on weather conditions. In the present study the orange fruits which were studied were from seedling trees, grown from seeds of the sweet orange. The fruit from these is commonly referred to as seedling oranges or seedlings.

The type of soil that the trees are grown in is sandy-clay which makes up 80% of the region with a pH of 6.5-7.

Environmental conditions of growth in terms of temperature, rainfall and RH are shown in Table 1. The minimum and maximum temperatures recorded were -5.6 and 30.2^{o}C respectively in 1980-81 with an average of 18.4^{o}C. In some exceptional years the temperature may drop to -13^{o}C (1962), -9^{o} (1970), and -7.5^{o}C (1972) and this causes serious damage to the crop.

The rainfall has been in the range of 19.0-266.4 mm in 1980-81 with an average of 989.4 mm. The RH was recorded to be in the range of 31-100% with an average of 79.6%.

Sampling of Material

The oranges were obtained from Rumsar Experimental Station in the Caspian region. In this grove plots of

Table 1.

The Climate in the Caspian Region

Month	Temperature (C^{O})						Relative Humidity (%)						Rainfall (mm)	
	1980			1981			1980			1981			1980	1981
	Min	Max	Ave	Min	Max	Ave	Min	Max	Ave	Min	Max	Ave		
Jan	2.0	6.4	8.9	-1.5	18.2	7.0	52.0	100.0	84.6	31.0	100.0	80.4	179.7	18.4
Feb	-1.5	19.0	5.7	-5.6	17.8	6.4	48.0	100.0	81.0	32.0	100.0	82.5	182.1	65.8
Mar	0.2	14.4	7.3	-3.0	20.5	7.3	46.0	100.0	84.5	34.0	100.0	79.1	67.0	86.0
Apr	0.6	20.6	11.1	1.0	26.6	10.6	53.0	100.0	86.5	43.0	100.0	84.2	119.3	82.7
May	9.5	25.0	16.5	12.6	23.9	17.3	56.0	100.0	84.8	42.0	100.0	88.2	103.2	29.9
June	12.5	17.5	19.9	12.0	28.0	20.4	62.0	100.0	79.2	57.0	100.0	82.1	51.4	52.0
July	17.0	30.2	23.9	14.8	30.2	23.6	56.0	98.0	78.8	56.0	98.0	81.1	31.7	8.9
Aug	18.2	31.0	25.1	16.2	29.4	23.5	47.0	98.0	79.2	52.0	100.0	81.5	39.0	24.5
Sept	18.1	30.5	25.5	15.6	30.1	23.4	56.0	100.0	84.4	51.0	100.0	79.6	62.5	84.7
Oct	10.0	25.0	18.2	9.0	26.0	16.5	60.0	100.0	86.0	59.0	100.0	81.2	120.2	266.4
Nov	5.8	24.2	15.1	3.2	20.0	12.7	44.0	100.0	81.9	55.0	100.0	83.4	19.0	252.5
Dec	2.6	23.0	12.1	-1.4	15.5	7.1	46.0	100.0	81.6	37.0	100.0	81.8	27.3	124.1

about 25 trees were selected. In the choice of plots care was taken to avoid abnormal soil and fertilizer practices. Exceptionally young and old trees were avoided. The trees (originally seedling trees) used were between 15-20 years old and were of known origins. Care was taken to pick only exposed fruits from the regular bloom.

Samples were usually collected at intervals of one month over a period of several months so that fruits of various stages of ripeness were obtained. The analyses were carried out while the fruits were still green and small in August and continued periodically until March when the fruits had reached their prime condition.

The fruit samples were air freighted one day after picking and reached London the following day when they were placed in storage at $3^{o}C$ until tested. The samples were tested within a few days of picking.

Statistical Analysis

The results of the experiments were analysed where appropriate statistically using the following formulae and statistical values as given by Steel and Torrie (1960) and shown below:

(1) The value of observations from the experiment

$(X_{i} \ldots\ldots_{n})$

(2) The mean number of observations for each treatment

$$\bar{x} = \frac{\Sigma X_i}{n}$$

n = number of replicates in each treatment

(3) Variance (s^2) of the mean

$$s^2 = \frac{\Sigma(X_i - \bar{x})^2}{(n - 1)}$$

(4) Standard deviation

$$s = \sqrt{s^2}$$

(5) Standard deviation of the mean (s_2)

$$s_{\bar{x}} = \sqrt{\frac{s^2}{n}}$$

(6) Student's t test

$$t = \frac{\bar{x}_1 - \bar{x}_2}{s_{\bar{x}_1} - s_{\bar{x}_2}} = \frac{\bar{d}}{s_{\bar{d}}} \quad \text{where}$$

$$s_{\bar{d}} = \sqrt{s^2_{\bar{x}_1} + s^2_{\bar{x}_2}} = \sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}$$

Calculated values of t were compared with published figures after obtaining the number of degrees of freedom.

(7) Tukey's w-procedure

$$w = q\alpha\,(p,\ n_2)s_{\bar{x}}$$

where $q\alpha$ is obtained from published figures for $\alpha = 0.05$, p is the number of treatments, n_2 equals error degrees of freedom, and $s_{\bar{x}}$ is standard deviation (error) of the means. w is used to judge the significance of each of the observed differences. Each difference is declared significant if it equals or exceeds the critical value of w, otherwise it is declared not significant. The mean values underscored by different letters indicate significant differences at 5% level of significance according to Tukey's w-procedure test. On the other hand, when the underscored letters are the same, the values are not significantly different at the 5% level.

Student's t test was used in experiments where two means were compared. In experiments where it was necessary to compare each treatment mean with every other mean (multiple comparisons) Tukey's w-procedure was used.

This procedure is very easy to apply since it requires a single value for judging the significance of all differences, that is, it is a fixed range; it takes into account the number of treatments in the experiment, and it permits decisions as to which differences are significant and which are not (Steel and Torrie, 1960). For example: Some Data taken from the experiment on seasonal changes on simple lipids of Sia-Varaz orange juice sacs (Table 8, p. 141) are given below:

Lipids	Sampling time (month)			
	Aug.	Sept.	Oct.	Nov.
MG	18.8 ± 0.9 ef	18.4 ± 0.9 e	19.2 ± 0.6 efg	20.0 ± 0.9 efgh
FS	66.7 ± 2.6 efg	64.0 ± 1.8 bcde	60.6 ± 1.7 bcd	46.0 ± 1.3 a
	Dec.	Jan.	Feb.	Mar.
MG	13.4 ± 0.6 cd	11.1 ± 0.4 c	6.8 ± 0.9 a	7.2 ± 0.2 ab
FS	52.8 ± 2.1 ab	57.4 ± 1.6 bc	66.6 ± 1.6 def	89.5 ± 3.0 h

From the figures above extracted from Table 8, it can be seen that there are a number of cases where both Tukey's w-procedure and standard error of the mean (s.e.) give the same probability, e.g. MG of

months September to November are not significantly different using either method. On the other hand, Tukey's test is more conservative, for when s.e. gives a significant result, Tukey's test does not; e.g. FS of months December and October.

This shows the conservative nature of Tukey's w-procedure. This is to be expected since Tukey's w-procedure requires that in 95% of one's experiments, differences are significant if the means are truly a homogeneous sample, and a single value is used for judging the significance of all observed differences.

Unless otherwise stated, the number of oranges per sample was 25.
However, the number of oranges used to produce the juice varied but was never less than 25. The extracted juice was pooled and required amounts were taken from this. So, in the early stages, hundreds of small oranges were used to produce the same volume of juice as 25 at the later stages.

All experiments were carried out at least three times and the results are the mean value of at least three replicates.

Please note that in a number of tables, the results are expressed as mg/100g or mg/100ml and the values given to one decimal point. Since 0. /mg/100g or /100ml is very small, it is therefore not significant in this context. So, although the values to one decimal point are shown in these tables, they are rounded off to the nearest integer, wherever possible, in the text.

Weight of Fruit

The oranges were weighed on a double pan balance. Samples of oranges were taken (approximately 25) and weighed. Average weight of one orange =

$$\frac{\text{weight of total oranges (g)}}{\text{number of oranges}}$$

Diameter of Fruit

The diameter of a sample of oranges was obtained by using calipers. Measurements were made at the equatorial points of the fruit.

Colour of Peel

The colour of the peel of oranges was determined by matching the fruit with the standards shown in Fig. 2; individual values were determined for 25 oranges, and from these an average was obtained.

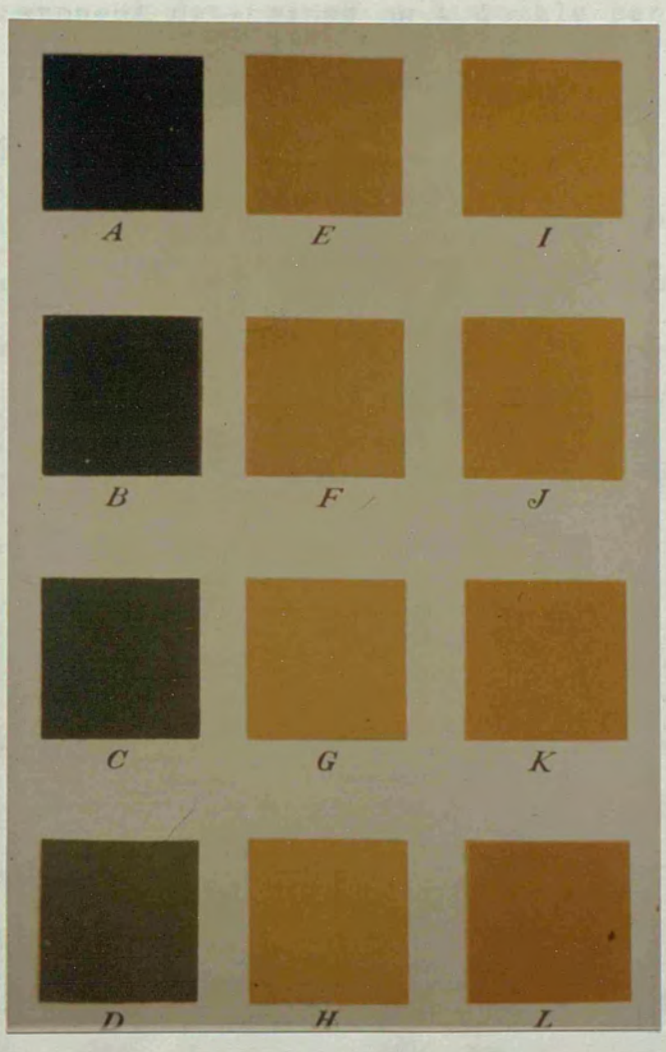
Thickness of Peel

Samples were cut in half transversely and the thickness of the peel measured by calipers.

Volume of Juice

The juice of orange fruits was extracted by hand-squeezing and then strained through a 30 mesh stainless steel sieve to remove pulp and seeds. The strained juice was transferred quantitatively to a measuring cylinder

Fig. 2. Colour chart used for determining the colour of the peel of orange (Harding et al., 1940)


A
E
I
B
F
J
C
G
K
D
H
L

for volume determination.

Weight of Component Parts

The component parts of fruits were carefully separated with the aid of a surgical knife and the weight of each component determined on a double pan balance.

Total Acidity

Total acid refers to titratable acidity, and was determined volumetrically by the amount of a standard alkali necessary to neutralize the acid in a known volume of orange juice (Hendrix et al., 1977). Braverman (1933) has stated that the acids in orange juice consist mainly of citric acid with small amounts of malic, tartaric and succinic acids.

Experimental

Ten ml of the juice sample was transferred to an Erlenmeyer flask followed by addition of 25 ml distilled water and 1 ml of phenolphthalein solution. This was titrated with 0.1 N sodium hydroxide to a faint pink colour (or pH 8.2) that lasted at least 30 seconds .

Acidity (g citric acid/100 ml juice) = v 0.063 where v = ml 0.1 N sodium hydroxide used in the titration.

pH Determination

The pH, or hydrogen-ion concentration, is a measure of active acidity. All the pH readings were carried out

on a PTI-11 Digital pH meter (Data Scientific, England) at 22°C.

Total Soluble Solids

The total soluble solids in orange juice are mainly sugars and organic acids which make up about 85% total. The remainder consists of inorganic compounds, and small amounts of pectins, essential oils, esters, glucosides and other organic compounds (Sinclair, 1961). These compounds are commercially important in that the occurrence of off-flavour in either fresh or processed orange juice is due to oxidation and decomposition of substances present in this relatively small fraction of soluble solids.

Total soluble solids were determined by the use of a "Bleeker" pocket refractometer (Nederlandsche Optik-en-Instrumentenfabriek, Zeist-Holland).

RESULTS AND DISCUSSION

Weight of Fruit

During the development and maturation period of the Sia-Varaz orange the weight of the fruit increased from 60.2 g in August to 178.2 g in March (Table 2). The results indicate that before the fruit reached its prime condition at the end of December the gain in weight was 99.9 g which was approximately a 25 g/month increase. From January to March the gain of 18.1 g corresponded to a 6.0 g/month increase. It may be observed (Fig. 3)

Table 2. Seasonal changes in the physical characters of the Sia-Varaz Orange

Sampling time (month)	Fr. wt./ fruit (g)	Diameter of fruit (mm)	Peel thickness (mm)	Juice/100 g of fruit (ml)	Colour of Peel
Aug	60.2 a	32.2 a	6.3 ef	3.0 a	Dark green(A)
Sept	72.4 ab	41.5 ab	5.5 de	7.2 ab	Green(C)
Oct	94.3 c	58.1 c	4.7 abcd	10.2 bc	Pale green(D)
Nov	122.6 d	62.4 cd	4.5 abc	25.6 d	Yellow green(E)
Dec	160.1 e	70.2 de	4.3 ab	32.7 e	Yellow(F)
Jan	171.1 ef	73.6 ef	4.2 a	38.0 ef	Orange(K)
Feb	177.8 efg	74.7 efgh	4.2 a	41.5 fgh	Orange(K)
Mar	178.2 fgh	74.1 efg	4.2 a	40.0 fg	Orange(K)

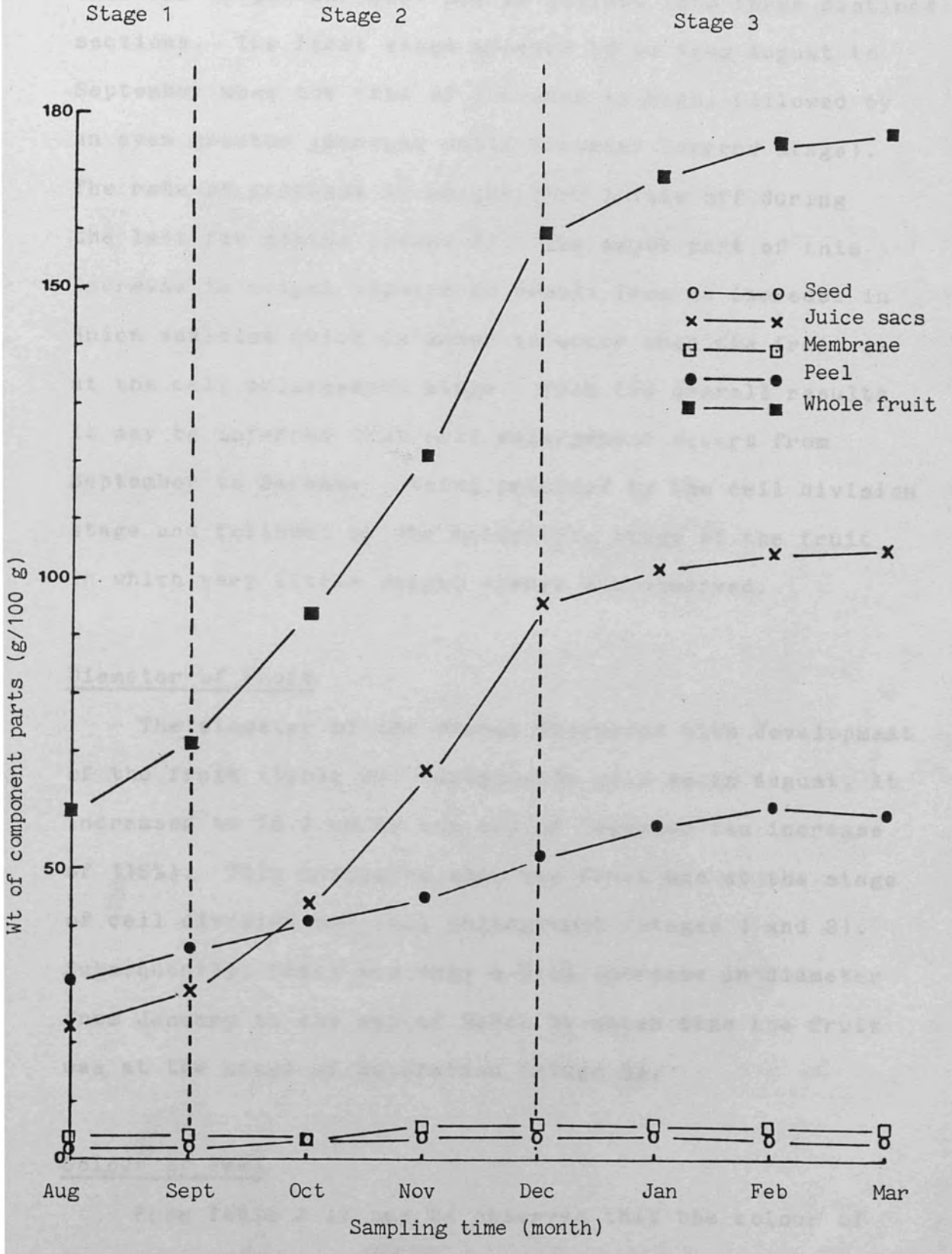


Fig. 3. Seasonal changes in the weight of whole Sia-Varaz Orange and their component parts

that the weight increase may be divided into three distinct sections. The first stage appears to be from August to September when the rate of increase is high, followed by an even greater increase until December (second stage). The rate of increase in weight then levels off during the last few months (stage 3). The major part of this increase in weight appears to result from an increase in juice vesicles which is known to occur when the fruit is at the cell enlargement stage. From the overall results it may be inferred that cell enlargement occurs from September to December, being preceded by the cell division stage and followed by the maturation stage of the fruit in which very little weight change was observed.

Diameter of Fruit

The diameter of the orange increased with development of the fruit (Table 2). Originally 32.2 mm in August, it increased to 70.2 mm by the end of December (an increase of 118%). This indicates that the fruit was at the stage of cell division and cell enlargement (stages 1 and 2). Subsequently, there was only a 5.6% increase in diameter from January to the end of March by which time the fruit was at the stage of maturation (stage 3).

Colour of Peel

From Table 2 it may be observed that the colour of the oranges remained in the green to yellow range from August to December. The transition to orange occurred between December and January, indicating the onset of

ripening.

Stearns and Young (1942) have shown that, in Florida, early and mid-season oranges changed from dark green to yellowish green only when the minimum temperature was between 7° and 13°C. During the colour transition for the Sia-Varaz oranges, between December and January the prevalent temperatures ranged from 7° - 12.1°C. This is in agreement with Stearns and Young's (1942) observation.

Weight of Component Parts

The results of weight determination of the component parts of the whole orange fruit are shown in Table 3 and Fig. 3.

The average weight of the fruit peel was 30.2 g/fruit representing 50.2% of the whole fruit in August. The weight of the peel showed a significant increase in the following months, reaching 60.0 g/fruit in March. As a percentage of whole fruit it showed a gradual but significant decrease during the development cycle, making up 33.7% of the fruit in March. The reason for these significant decreases (on a percentage basis) was the substantial increase in the weight of juice sacs.

The initial weight of juice sacs was 23.7 g/fruit making up 39.4% of the whole fruit in August. The weight of this component part of the fruit showed little change for the first two months, indicating the cell enlargement

Table 3. Seasonal changes in the component parts of the Sia-Varaz orange fruit. Graphical presentation of the data is shown in Fig. 3.

Sampling time (month)	Fresh weight/ fruit (g)	Component Parts							
		Weight (g)				Percentage			
		Peel	Juice sacs	Membrane	Seeds	Peel	Juice sacs	Membrane	Seeds
Aug	60.2 a	30.2 a	23.7 a	4.2 a	2.1 a	50.2	39.4	7.0	3.5
Sept	72.4 ab	36.4 ab	28.8 ab	4.4 ab	2.8 b	50.3	39.8	6.1	3.9
Oct	94.3 c	41.7 bc	44.2 c	4.8 abc	3.6 c	44.2	46.9	5.1	3.8
Nov	122.3 d	45.6 cd	67.1 d	5.6 d	4.0 cd	37.3	54.9	4.6	3.3
Dec	160.1 e	53.3 e	96.2 e	6.1 de	4.5 de	33.3	60.1	3.8	2.8
Jan	171.1 ef	56.2 ef	102.3 ef	6.5 ef	5.1 efgh	33.4	59.8	3.8	3.0
Feb	177.8 efg	60.6 efg	105.6 efg	6.7 efgh	4.9 efg	34.1	59.4	3.8	2.7
Mar	178.2 fgh	60.2 efgh	106.6 fgh	6.6 efg	4.8 ef	33.8	59.8	3.7	2.7

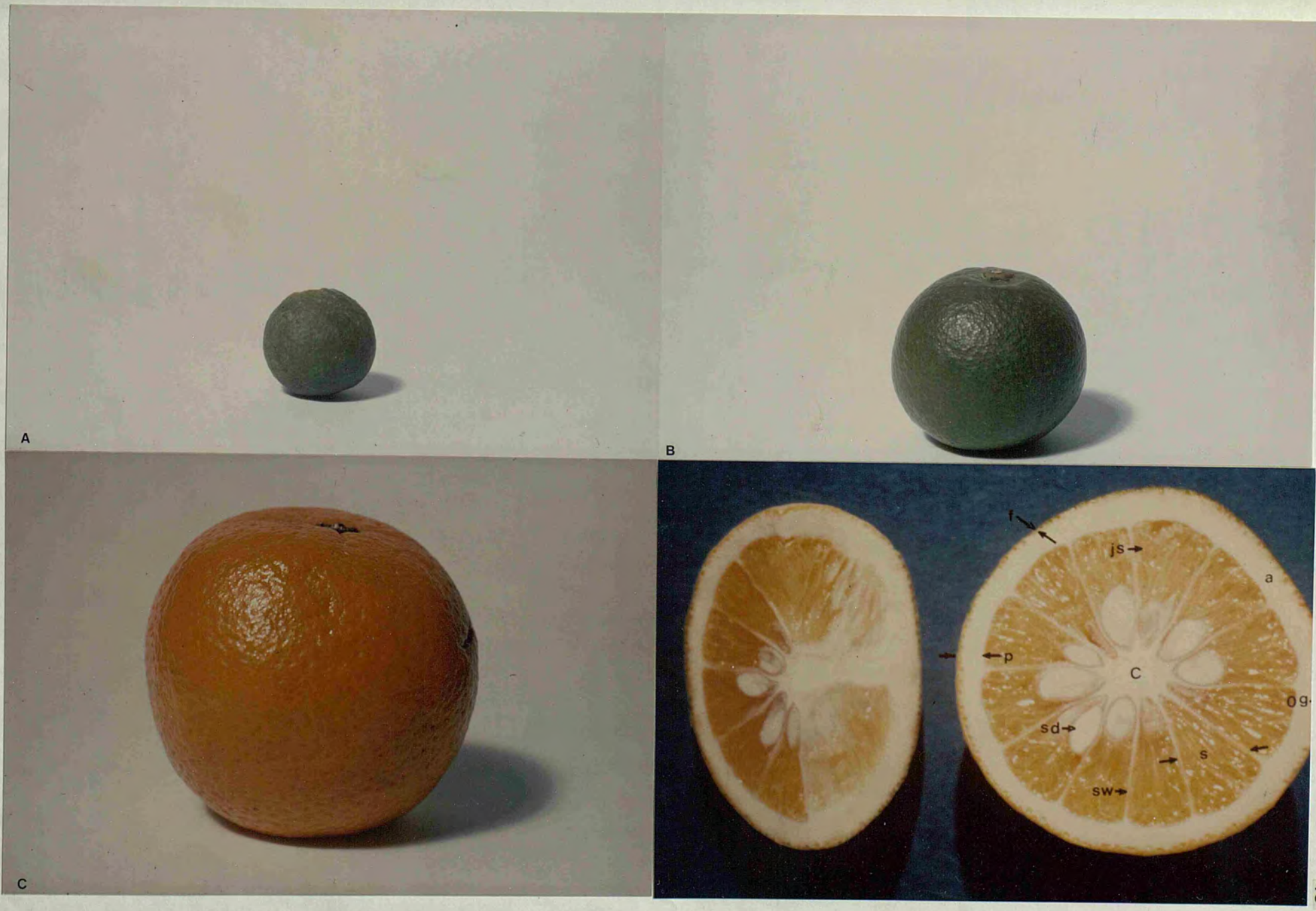
stage in the fruit development cycle. Between October and January there was a significant increase both in weight and in percentage followed by a very small increase in weight and percentage in the last three months of fruit development. The low weight of juice sacs in the first two months and its significant increase between October and January followed by a levelling-off in February and March, in fact indicated the three stages of the whole development cycle of the fruit. The results of the above, and those of section 1 indicated that most of the changes taking place during maturation (stage 3) were chemical rather than physical. An interesting fact emerged from finding the weight of juice sacs in that the significant increase in weight of the whole fruit was mostly due to the substantial increase in the weight of juce sacs as they enlarged and became filled with juice.

Membrane or sections walls (Fig. 4) showed a significant increase in weight between stages 1 and 2, but not afterwards. As a percentage of the weight of the whole fruit it showed a significant decrease during the cell enlargement period (stage 2) and with no marked changes in the maturation period.

The Sia-Varaz orange fruit is a many-seeded variety. The number of seeds found in individual fruits varied between 12 and 30.

The average weight of the seeds in the whole fruit

Fig. 4. Seasonal changes in size and colour of the Sia-Varaz orange (A, B, C). Also, equatorial sections of the fruit (D) are shown. Distinguished parts of the fruit discussed in the text include: peel (p); flavedo (f); albedo (a); oil glands (og); sections (s); segment wall (sw); juice sacs (js); seeds (sd); and core (c)


A
B
C
f
js
a
p
C
og
sd
s
sw

was 2.1 g in August representing 3.5% of fruit weight. Between August and January it showed a significant increase and reached 5.2 g/fruit in January. From January to March it showed a small decrease in weight.

Volume of Juice

Table 2 shows that very little juice could be extracted from oranges in August and September (3 ml/100 g fruit). However, between October and December, there was an average increase of 10 ml/100 g per month which was reduced to an average of 3 ml/100 g per month after this period.

From the results of the increase in diameter of the fruit (discussed on p.44) it was assumed that stages 1 and 2 of development ranged from August to December which supported the results discussed previously. The fact that maximum juice production occurred between October and December is further support for what has just been shown, that cell enlargement occurred between these months.

Total Acidity

The total acid content of Sia-Varaz orange juice decreased from 2.3 g/100 ml in August to 0.8 g/100 ml in March, indicating a downward trend (Table 4 and Fig. 5). A close look at the results shows that maximum decrease occurred between October and December during the stage of cell enlargement. This significant decrease in total acidity may have been caused by a dilution effect due to juice formation.

Table 4. Seasonal changes in the chemical constituents of Sia-Varaz oranges. Graphical presentation of the data is shown in Fig. 5

Sampling time (month)	pH	Total acid (g/100 ml)	Total soluble solids (g/100 ml)	Total soluble solids/acid ratio of juice
Aug	2.5 a	2.3 gh	7 a	3.0 a
Sept	2.7 ab	2.1 fg	8 ab	3.8 ab
Oct	2.9 c	1.9 ef	9 abc	4.7 c
Nov	3.1 cd	1.7 e	10 abcd	5.9 d
Dec	3.2 de	1.3 cd	12 cde	9.2 e
Jan	3.3 ef	1.1 abc	13 def	11.8 f
Feb	3.7 g	0.9 ab	13 def	14.4 g
Mar	3.8 gh	0.8 a	13 defg	16.3 h

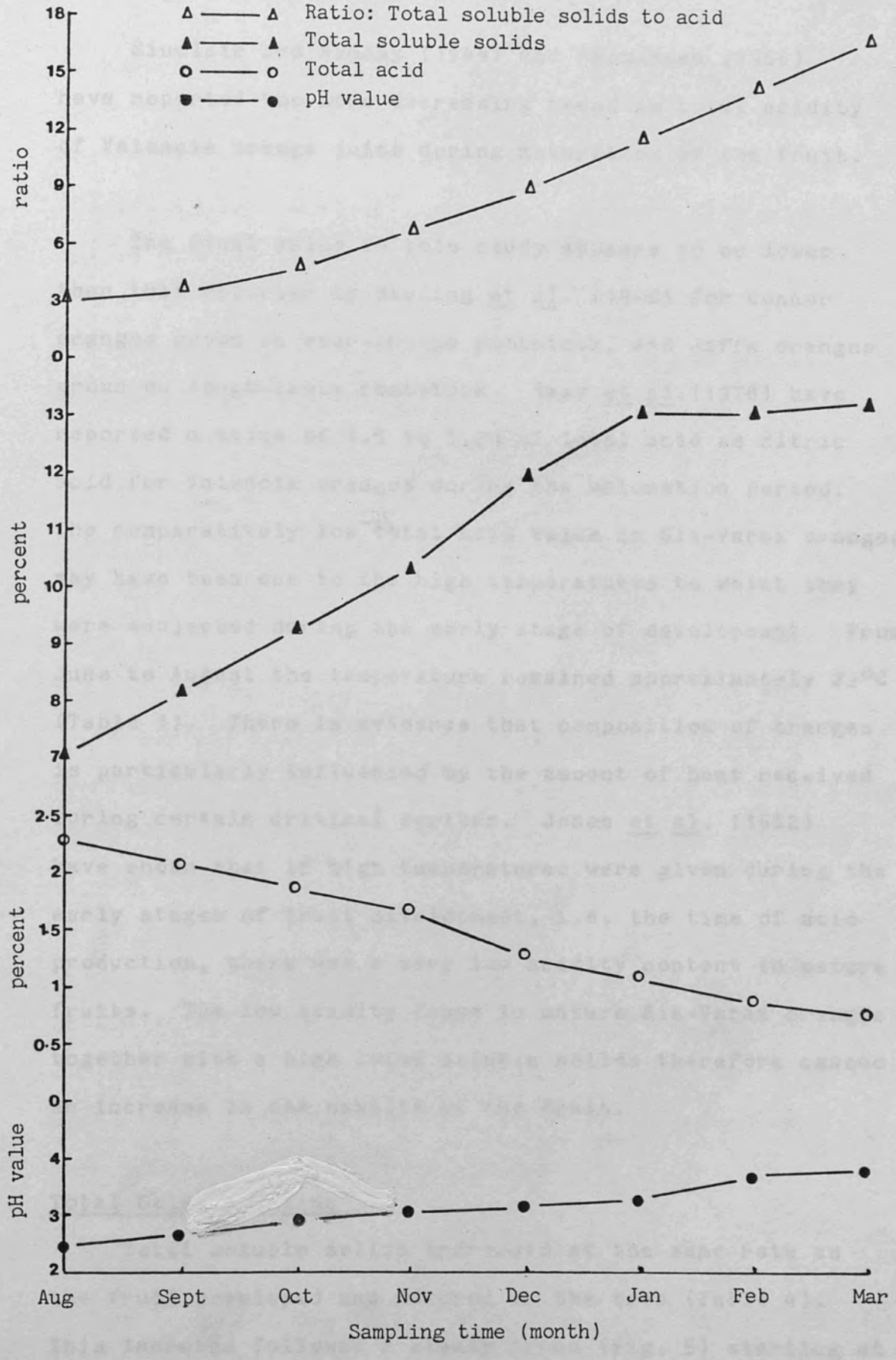


Fig. 5. Seasonal changes in the chemical composition of the Sia-Varaz orange juice. For significant differences refer to Table 4

Sinclair and Ramsay (1944) and Rasmussen (1966) have reported the same decreasing trend in total acidity of Valencia orange juice during maturation of the fruit.

The final value in this study appears to be lower than that reported by Harding et al. (1940) for Conner oranges grown on sour-orange rootstock, and Jaffa oranges grown on rough-lemon rootstock. Nagy et al.(1978) have reported a value of 1.5 to 1.2% of total acid as citric acid for Valencia oranges during the maturation period. The comparatively low total acid value in Sia-Varaz oranges may have been due to the high temperatures to which they were subjected during the early stage of development. From June to August the temperature remained approximately 23°C (Table 1). There is evidence that composition of oranges is particularly influenced by the amount of heat received during certain critical periods. Jones et al. (1962) have shown that if high temperatures were given during the early stages of fruit development, i.e. the time of acid production, there was a very low acidity content in mature fruits. The low acidity found in mature Sia-Varaz oranges together with a high total soluble solids therefore caused an increase in the quality of the fruit.

Total Soluble Solids

Total soluble solids increased at the same rate as the fruit developed and matured on the tree (Table 4). This increase followed a steady trend (Fig. 5) starting at 7 and reaching a value of 10 g/100 ml by November,

corresponding to an average increase of 1g/100 ml per month. During December there was a rapid total soluble solid accumulation amounting to 2 g/100 ml whereas only a total of 1g/100 ml gain was recorded in the next three months of development.

This increase was mostly due to increased synthesis of sugars in the fruit which makes up approximately 80% of the total soluble solids in the mature orange.

Ratio of Total Soluble Solids to Total Acids

The soluble solids and the total acidity of orange juice are closely associated with the edible quality of the fruit. The increase in total soluble solids as the fruit matures with a corresponding decrease in total acids, has resulted in the use of the ratio of total soluble solids to acid as a measure of maturity for the commercial picking of the fruit.

In the present study the soluble solids to acid ratio of the juice increased with maturity of the fruit (Table 4 and Fig. 5). This increase in the ratio was due to a corresponding decrease in total acidity coupled with a large increase in the sugar content.

The ratio for the immature fruit was low (3.0) but rose steadily to 5.9 by November, implying that the juice was still sour. By December, however, this ratio rose steeply to 9.2 indicating that the juice was approaching

the acceptable limits of preference in terms of taste. Mayo and Taylor (1936) have mentioned that a fruit was deemed mature only when the ratio of total solids of the juice to the acid content was greater than 8:1. Regarding this, Taylor (1938) stated that the 8:1 ratio was too low for early oranges.

From the results in this study, and using the above limit as a criterion, it would be expected that, by December, the orange would be deemed mature, since the ratio was 9.2:1. However, reports from Iran (1964) have indicated that this ratio for Iranian sweet oranges should be 10:1. This would imply that the Sia-Varaz orange would be officially termed mature for picking in January. This coincides with the inference made earlier, as a result of determining the weight of the orange, that the maturation cycle is initiated between December and January.

Another criterion for maturity was that adopted by the Florida Citrus Growers (Rose and Henry, 1912) who decided that the percentage weight of total sugar must be seven times greater than the weight of total acids for the fruit to be deemed mature.

From results to be discussed later (p. 65) it was calculated that the sugar to acid ratio reached a value greater than 7:1 in January.

CONCLUSION

From the results of Tables 2 and 4, it can be seen that the stages 1, 2 and 3 in the development and maturation cycle of the Sia-Varaz orange are easily distinguishable: cell division occurs mainly in August and September, cell enlargement between October and December and maturation occurs from December to March.

The main parameters which would determine the optimum time for the harvesting of the oranges for direct consumption or subsequent processing include the weight of the whole orange, the peel colour, the volume of the juice, and the ratio of total soluble solids to acid.

The colour of the fruit remained unchanged throughout maturation implying that the orange may be harvested any time between January and March. From a commercial point of view, March would seem the best time for selling the oranges as their weight would be greatest then and hence would fetch a higher price on a wholesale basis.

However, the attractiveness of the fruit to the consumer must play a great part in its sale. This depends greatly upon the balance between sweetness and acidity which is determined by the total soluble solids to acid ratio. A low ratio means that a fruit or juice is relatively sour while a high ratio means that it is relatively sweet. Bayton and Bell (1954) studying

consumer preferences found that those who generally liked juices preferred a ratio of between 12 and 18:1.

The stage of development in the present study which reflects these ratios most closely were between February and March

It is suggested that the best time to harvest Sia-Varaz oranges is any time in March.

CHAPTER II

SEASONAL CHANGES IN FREE SUGARS

INTRODUCTION

In citrus fruits soluble carbohydrates (sugars) and insoluble carbohydrates (polysaccharides) dominate the solid content (McCready, 1977). The main carbohydrates of orange fruits are the three sugars, sucrose, glucose and fructose (McCready et al., 1950). Together they represent about 80% of the total solids (Bartholomew and Sinclair, 1943), and the ratios of sucrose:glucose:fructose are generally about 2:1:1 in the mature orange juice (Curl and Veldhuis, 1948; Ting and Attaway, 1971). McCready et al. (1950), using paper chromatography, identified these three sugars to be the main ones in the juice of Florida Valencia oranges, while Alberola et al. (1967) identified α- and β-glucose, fructose, sucrose and a small amount of galactose in Valencia orange juice after the use of gas-chromatography. The sweet taste of the mature orange pulp and the flavour of the juice is due to a blend of sugars, acids and specific flavour compounds (McCready, 1970).

During the development stage the fruits will be reaching their full size and during ripening the fruits will become edible and desirable as food. Certain changes are occurring in the quality and quantity of sugars within the fruit. Citrus fruits, unlike starchy fruits such as apples and

pears, contain practically no starch and, consequently, do not undergo any change in sugar composition after being detached from the tree (Braverman, 1933). The content of the sugars in the orange fruits may vary on a variety of factors such as rootstock (Gardner and Horanic, 1967), fruit size (Smith, 1967; Marloth and Basson, 1959), growth regulators (Monselise and Goren, 1965), climate (Koo, 1963; Cooper et al., 1963), and stage of maturity.

It was therefore important to study the sugar content of Sia-Varaz oranges at different stages of maturity and to observe differences, if any, with that of other well-known orange varieties.

MATERIALS AND METHODS

Quantitative Determination of Free Sugars by GLC Separation of TMS Derivatives

The fact that free sugars are generally soluble in aqueous alcoholic solutions while proteins and most polysaccharides are not at certain alcoholic strengths is made use of in the purification of free sugars. The alcoholic extracts, however, are sometimes contaminated by some lipids, pigments and free amino- and organic-acids in the sample. The next stage in the preparation of the extract must be to remove these interfering substances. Addition of saturated lead acetate precipitates most pigments and organic acids.

Separation of sugar derivatives by GLC is now well established. Sweeley *et al*. (1963) have shown that trimethylsilyl (TMS) derivatives of sugars are suitable for the quantitative and qualitative analyses of sugar mixtures.

Sugars are crystalline polyhydroxy compounds which are strongly hydrogen bonded, hence they have high melting points (200-300^{o}C) (Birch, 1973). They can be converted into volatile derivatives such as trimethylsilyl ethers by reacting with trimethylsilyl chloride (TMSCl). The TMS ethers (Fig. 6) have the combined advantage of stability

and volatility which allows the separation of mono, di- and tri-saccharides.

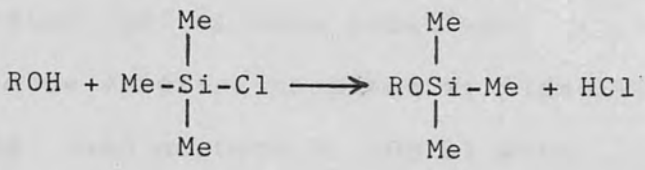


Fig. 6. Simplified scheme of formation of trimethylsilyl derivatives of sugars

Mixtures of (TMS) derivatives of sugars may be separated readily by GLC, especially when this has facility for temperature programming of the column.

Taking the above consideration into account the technique used by Kline et al. (1970) was chosen for the identification and quantitative determination of sugars in orange juice.

1. Apparatus and Reagents

(a) Gas chromatograph

Pye (series 104) equipped with a hydrogen flame ionisation detector, nitrogen carrier gas and full-scale drift per hour of 10^{-12} was used. Glass column (1.5 m long and 2 mm in diameter) was packed with 4% OV-17 on gas chrom Q, 100-120 mesh, supplied by Camlab England. Gas chromatograph was operated with H_2 pressure of 12 p.s.i., air pressure of 10 p.s.i. and nitrogen flow of 50 ml min^{-1}. Temperature programmed from

220 to 270°C at 4°C min^{-1}. Chart speed of 25.4 cm h^{-1} and attenuation of 5 x 10^{-5}.

(b) Saturated lead acetate solution

Saturated solution prepared by dissolving ca. 16 g neutral lead acetate in 100 ml water.

(c) Drierite ($CaSO_4$) supplied by Koch-Light Laboratories Ltd.Eng. Anhydrous, 8 mesh.

(d) Celite.

(e) Internal standard

α - D Glucoheptose.

(f) TMS reagent

25 ml vial of Tri-Sil (Pierce Chemical Co.).

(g) Sugars

Purified.

2. Sample Preparation

(a) Orange juice

One hundred ml of orange juice was weighed into a 500 ml volumetric flask and diluted to volume with 80% ethanol. This was mixed thoroughly and left to stand for at least 1 hour.

(b) Whole fruit

One hundred g of orange pulp was weighed and transferred to a blender; 300 ml of 80% ethanol was added and blended for 15 minutes. This was then transferred quantitatively to a 500 ml volumetric flask and diluted to volume with 80% ethanol. The whole was mixed thoroughly and left to stand for at least 1 hour.

3. Sugar Determination of Samples

Ten ml aliquot of the prepared filtered sample was transferred to a graduated centrifuge tube. Saturated lead acetate (0.5 ml) was added to the tube to precipitate polybasic acids. The tube was capped, shaken thoroughly, and left to stand for 15 minutes. This was then centrifuged and the clear supernatant was decanted into a vial. 1.05 ml aliquot was pipetted into a vial and approximately 0.1 g Celite added. This was swirled and then evaporated to complete dryness in a vacuum desiccator at 30°C for 48 hours. Approximately 5 mg of the internal standard was weighed accurately and added into the vial. A few granules of Drierite were added and 2.0 ml TMS reagent injected from a hypodermic syringe. The teflon-lined screw-cap vial was closed tightly and shaken thoroughly to mix the residue with the solution. The solution was left to stand for 30 minutes at 45°C when the reaction was complete. The solution was left at room temperature to settle out and 1 µl of the clear solution was injected into the gas chromatograph. (The sample size depended on concentration and sensitivity was adjusted in order to keep the major peaks on the scale).

4. Preparation and Determination of Standards

One hundred and twentyfive mg each of the following selected pure sugars were dissolved in 20 ml 80% ethanol: D-fructose, D-glucose, D-galactose, xylose and sucrose. These were diluted to volume in a 25 ml volumetric flask, each solution containing 5 mg of each sugar/ml. One ml aliquot

was transferred to a vial, 0.1 g Celite added and the procedure as outlined under "sugar determination of samples" (page 63) carried out and 5 mg internal standard added.

0.5 µl standard mixture was injected into the gas chromatograph and from the peaks obtained it was possible to estimate quantitatively the sugars present in the sample.

5. Calculations

The relative response factor (RRF) was calculated so that the peak area of each sugar was equivalent to that of the internal standard.

RRF = Peak area of internal standard/peak area of sugar.

Two peaks appeared in the case of glucose, so that glucose peak area was the sum of the α - and β - peaks. The amounts of each sugar in fruits and juices were calculated as follows:

$$\% \text{ known sugar} = A_s \times RRF \times W \times 100/(A_i \times S)$$

Where A_s and A_i = peak area of known sugar in sample and internal standard respectively; RRF = relative response factor of known sugar; W = mg internal standard added; S = Vol. sample.

Since xylose has not previously been reported in orange juice a further experiment with TLC was carried out to verify its presence.

Sample Preparation

Preparation of samples was the same as described on pages 62 and 63 except that the procedure was completed by adding saturated lead acetate followed by centrifugation.

Sugar standards Preparation

Preparation of sugar standards was the same as described on Page 63.

TLC Procedure

The sugars were separated and identified by thin-layer chromatography according to the method of (De Stefanis and Ponte, 1968). In this procedure a thin-layer plate (20 x 20 cm) plastic backed, coated with a layer of 0.25 mm Silica gel G, type polygram Sil G, supplied by Macherey Nagel and Co. Ltd., was ruled in 10 mm wide lanes with a space of 0.5 cm between them. The unknown sugar sample from orange juice was applied to the plate with a microsyringe alongside the standards as well as co-chromatographed with them. Applications were made in small increments (less than 2µl)under a stream of warm air to facilitate rapid drying, and thus to minimize spot diffusion.

The plate was rolled into a 250 ml beaker and the beaker was inverted within the chromatography tank. The 'inverted' technique maintained a constant concentration of solvent vapour across the surface of the plate and reduced the bowing of the solvent front (Menzies et al., 1978).

The plate was developed with the solvent system: Chloroform-acetic acid-water (3.0:3.5:0.5 v/v) right to the top of the plate, and was air dried over night to remove traces of solvents.

Detection of spots

The reagent used for detection of sugars was made up by dissolving 1 g diphenylamine and 1 ml aniline in 100 ml of acetone; prior to spraying, 10 ml of the acetone solution was mixed with 1 ml of 85% phosphoric acid. The sprayed plate was heated for 10 minutes at 130°C. Most of the sugars appeared as dark grey spots; xylose, however, was light bluish-grey, and fructose was rosy brown (when freshly heated) (Fig. 6A).

The results (Fig. 6A) show that the unknown sugar co-chromatographs with xylose and therefore, it seems likely that xylose is present in the immature orange juice.

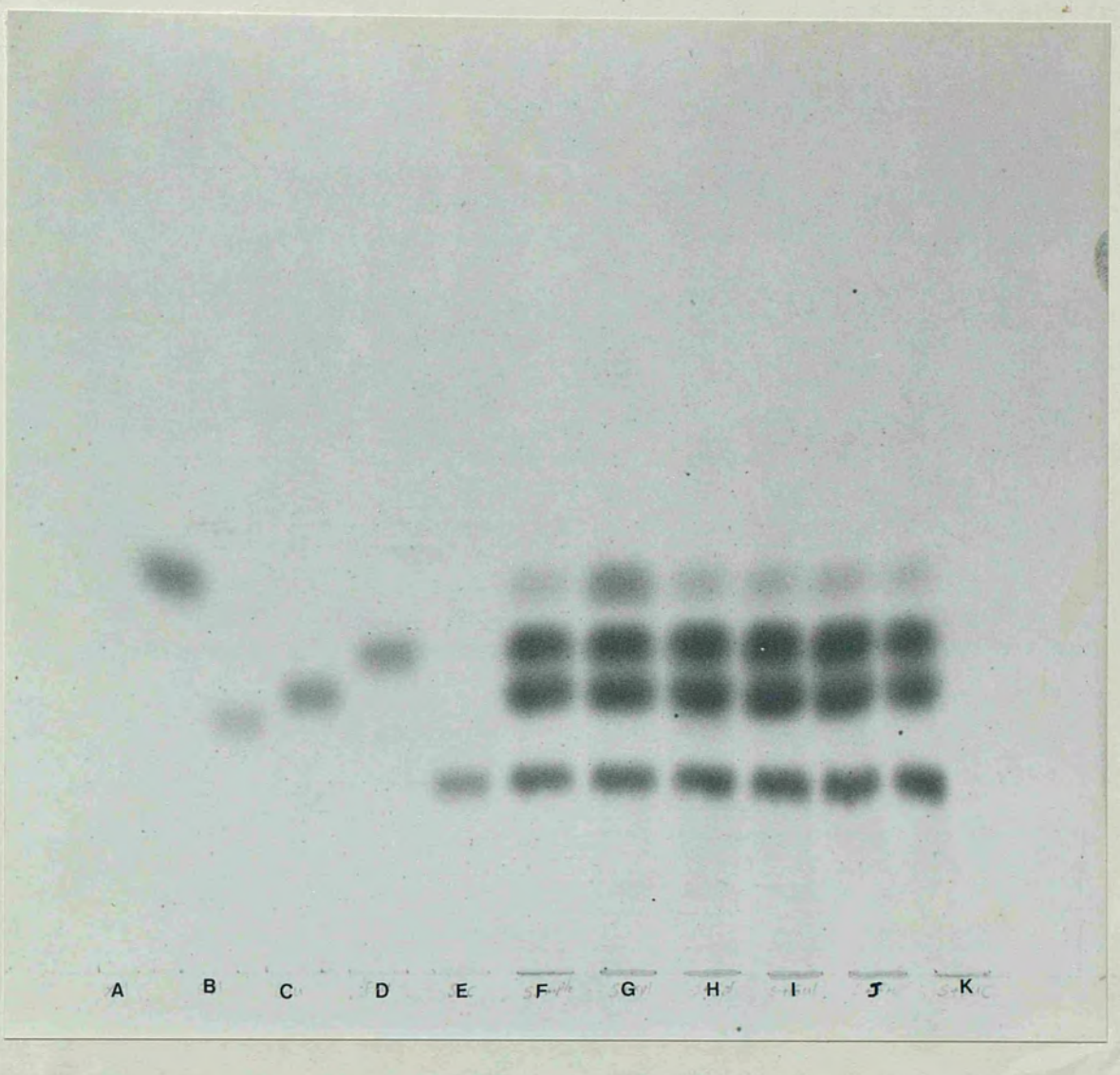


Fig. 6A Separation of free sugars by TLC.

A = Xylose; B= Galactose; C = Glucose;

D = Fructose; E = Sucrose; F = Sample;

G = Sample + Xylose; H = Sample + Galactose;

I = Sample + Glucose; J = Sample + Fructose;

K = Sample + Sucrose.

Solvent : Chloroform - acetic acid - water

(3.0 : 3.5 : 0.5 v/v).

RESULTS AND DISCUSSION

Table 5 and Fig. 7 show the concentration of free sugars in different stages of fruit development. Four sugars, namely sucrose (Suc), glucose (Glu), fructose (Fru) and galactose (Gal) were identified (Fig. 8), similar to the results obtained by Alberola *et al*. (1967). Xylose (Xyl) was also identified but was only found in the early samples (August and November) when the fruits were immature. This is the first reported instance of xylose being identified in orange juice. The reason that it had not been reported earlier may have been due to the fact that it was found in small amounts and then only at the immature stages. The results (Table 5) also indicated that glucose concentration did not increase significantly from its initial value in August (1.0 g/100 ml) until November, but increased substantially to 1.9 and 2.4 g/100 ml in December and January respectively. The latter value showed no significant change in February and March. A similar increasing trend was observed with fructose.

In the present experiment the initial sucrose concentration was higher than that of glucose or fructose, and its concentration did not change substantially for the first two months. Its content increased significantly to 3.2 and 3.9 g/100 ml in October and November respectively. For the next four months it showed no significant change. However, the increase in concentration of glucose and fructose increased with maturity to a maximum of 2.6 and

Table 5. Seasonal changes in the free sugars. Graphical presentation of the data is shown in Fig. 8

Sampling time (month)	Free sugars (g/100 ml)					
	Xylose	Glucose	Fructose	Galactose	Sucrose	Total
Aug	0.3	1.0 a	1.1 a	0.4 b	1.9 a	4.7 a
Sept	0.2	1.1 ab	1.2 ab	0.5 bc	2.3 ab	5.3 ab
Oct	-	1.2 abc	1.5 abc	0.6 cde	3.2 bc	6.5 bc
Nov	-	1.4 abcd	1.8 bcd	0.7 efg	3.9 d	7.8 cd
Dec	-	1.9 de	2.4 de	0.7 efgh	4.3 de	9.3 e
Jan	-	2.4 ef	2.8 ef	0.6 cdef	4.6 ef	10.4 ef
Feb	-	2.5 efg	2.7 efg	0.5 bcd	4.8 efg	10.5 efg
Mar	-	2.6 fgh	2.8 efgh	0.2 a	5.1 fgh	10.7 efgh

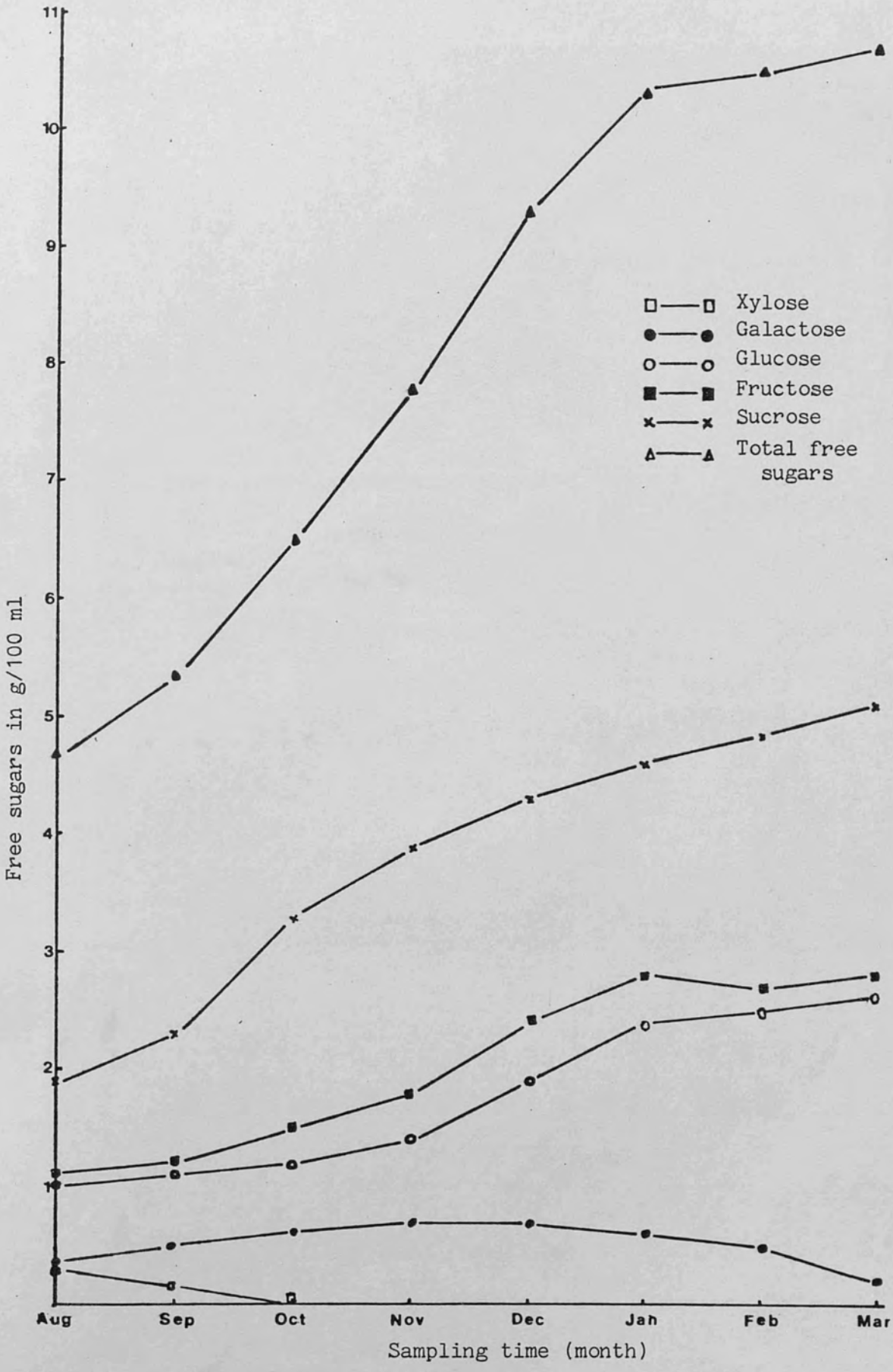
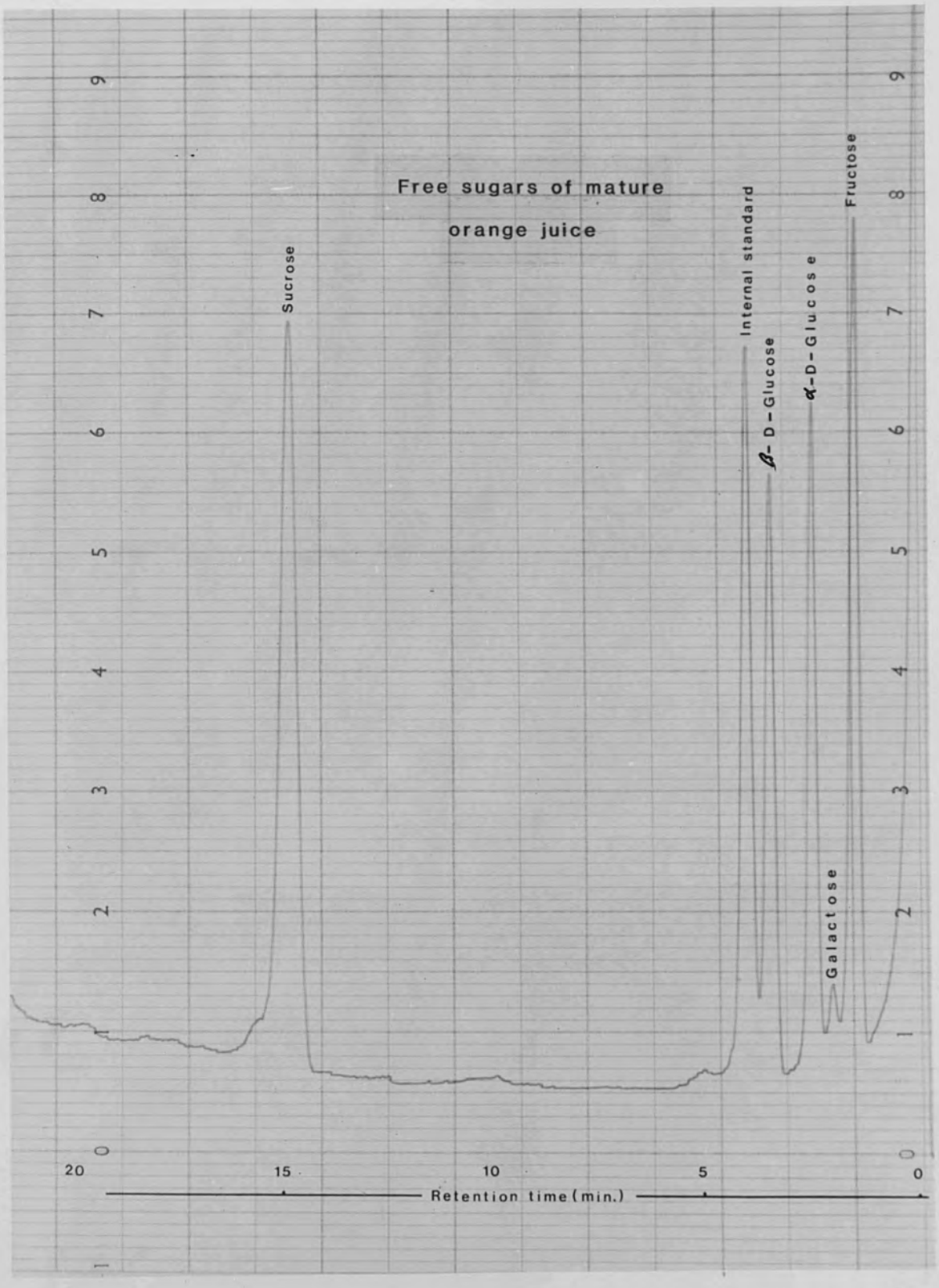


Fig. 7. Seasonal changes in the free sugar contents of the Sia-Varaz orange juice. For significant differences refer to Table 5

Fig. 8. Gas-liquid chromatography of free sugar methyl esters on a 150 cm 4% OV-17 column with temperature programmed from 220 to 270°C at 4°C min^{-1}


Free sugars of mature
orange juice
Sucrose
Internal standard
β-D-Glucose
α-D-Glucose
Fructose
Galactose
20
15
10
5
0
Retention time (min.)

2.8 g/100 ml respectively, which were approximately half the levels of sucrose (5.1 g/100 ml) in the March samples. Sucrose remained the major single sugar throughout the season. Galactose was another monosaccharide found in Sia-Varaz orange juice. The concentration of galactose in early season samples was 0.4 g/100 ml and this increased to a maximum level of 0.7 g/100 ml in November and December, then decreased significantly and reached a negligible but measurable amount by March. The presence of galactose in such small amounts does not contribute to the flavour or to the nutritional value of the mature orange juice and seems therefore to be of minor importance.

In general, the present results agree with those reported by several other workers (Collison, 1913; Harding et al., 1940; Ting and Attaway, 1971) in that increases in total free sugars were due to a gradual increase in both sucrose and reducing sugars. They differ however from those obtained by Braverman and Carmi (1937), who found that in Palestine oranges the reducing sugars remained practically constant during the season and that the increase in total free sugars was mainly due to an increase in sucrose.

The increase in free sugars during cell division (August-September) ranged from 9% for fructose to 25% for galactose. However, maximum increase in sugars occurred during the stage of cell enlargement (October-December), ranging from 40% in galactose to 100% in fructose. The

percentage increase of sucrose during maturation (January-March) again dropped to approximately 10%. This suggested that the rate of sugar synthesis was at its maximum during the stage of cell enlargement as indicated by the 75% increase in total free sugars during this period. Further support was obtained from the fact that free sugars were significantly increasing between the months of October and November, coinciding with the initiation of cell enlargement (p. 44).

In the present study, total free sugars of mature Sia-Varaz oranges were 10.5 g/100 ml which are higher or equal to those reported for Valencia (8.7 g/100 ml) (Harding et al., 1940), for Hamlin (9.0 g/100 ml) and Pineapple (10.5 g/100 ml) (Ting, 1954). This makes the Sia-Varaz orange juice a useful contributor for blending with other juices in order to enhance their sugar content.

CHAPTER III

SEASONAL CHANGES IN VITAMIN C

INTRODUCTION

The most important contribution of citrus fruits to human nutrition is attributed to their high vitamin C content. Although citrus fruits and their products are not the only source of high content of vitamin C among fruits and vegetables, their popularity is largely due to their desirable flavour, taste and colour.

As in other plant tissues, fruits synthesize vitamin C from hexose sugar precursors. In the first instance the synthesis depends on an adequate supply of hexose sugars, and thus on photosynthetic activity. As would be expected, a decrease in the photosynthetic activity induced by reducing light intensity is reflected in a decreased level of vitamin C. With fruits, therefore, the concentration of vitamin C varies with the degree to which the fruit is exposed to sunlight (McCollum, 1944). Conversely, a fall in the level of vitamin C may be observed when parts of the plant are shaded (Brown and Moser, 1941; Wokes and Organ, 1943).

The connection between the formation of hexose sugars and synthesis of vitamin C has been reviewed by Mapson (1967). In general, two pathways have been proposed for the synthesis in plants and animals, both originating from

either D-glucose or D-galactose as outlined in Fig. 9.

The vitamin C content of orange fruits and their products are affected by (1) production factors and climate (Smith, 1969), (2) maturity state and position of fruit on the tree (Harding et al., 1940; Sites and Reitz, 1950b), (3) type of orange fruit (Harding et al., 1940; Hutchison and Hearn, 1977).

The effect of development and maturation on vitamin C content have been studied by Atkins et al. (1945). Their results indicate a continuous decrease of vitamin C level during maturation. Harding et al. (1940) observed that vitamin C concentration was high in immature orange fruits and decreased as they ripened. Generally speaking, mature oranges contain from 40-70 mg vitamin C per 100 ml of juice (Ting and Attaway, 1971). In order to fully understand the changes that occur during the final stages of maturation and post-harvest, it is important to know the vitamin C content during the early stages of development and maturation of the pulp of the fruit.

In the present study the changes in vitamin C with development and maturation were investigated.

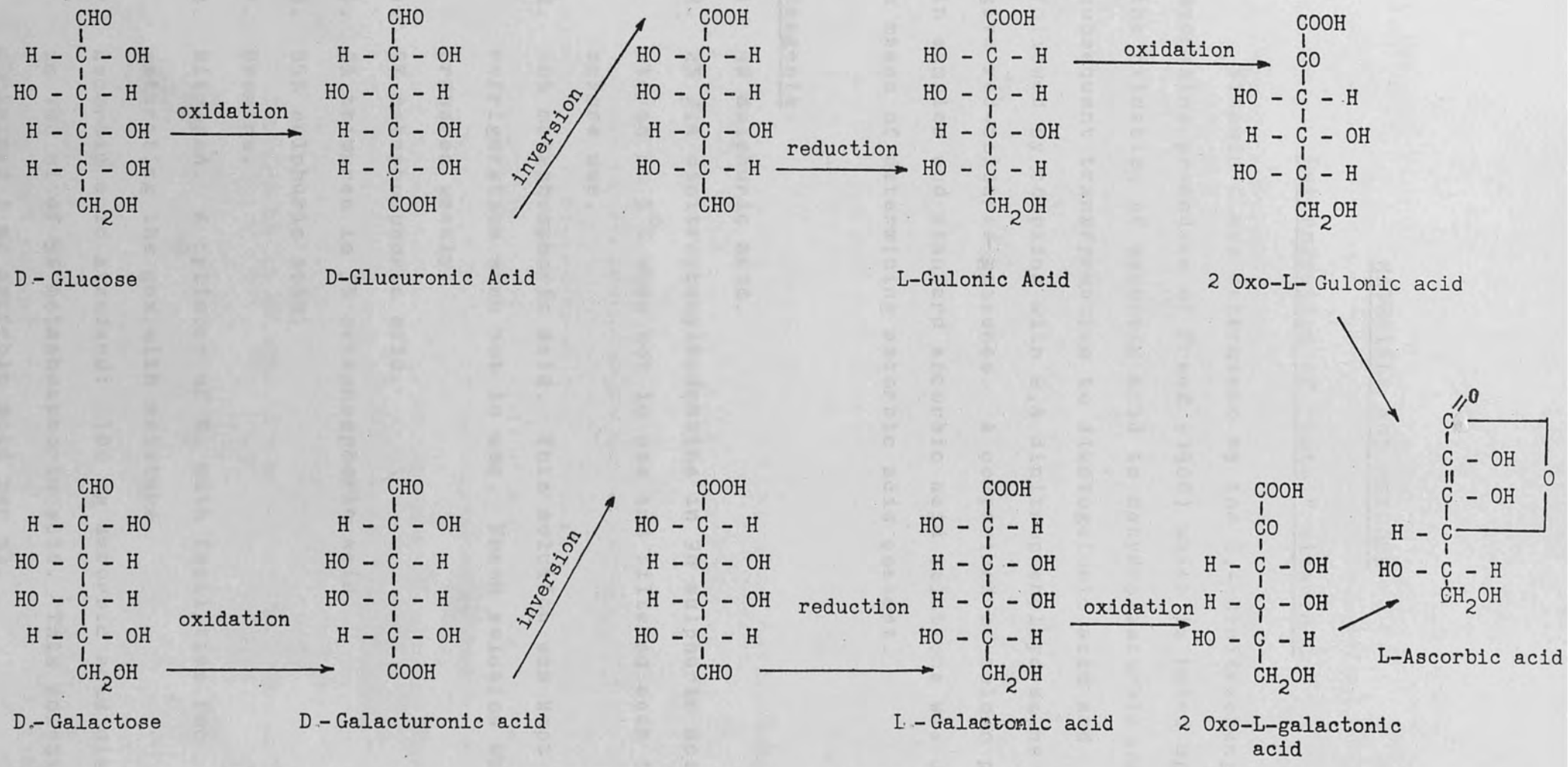


Fig. 9. Pathways in the conversion of D-glucose or D-galactose to L-ascorbic acid

MATERIALS AND METHODS

Determination of "total" vitamin C

Vitamin C was determined by the 2,4 dinitrophenylhydrazine procedure of Freed (1966) which is based upon the oxidation of ascorbic acid to dehydro-ascorbic and its subsequent transformation to diketogulonic acid and followed by coupling with 2,4 dinitrophenylhydrazine to give red-coloured osazones. A comparison of colour produced in samples and standard ascorbic acid solutions was used as a means of determining ascorbic acid content.

Reagents

1. 9N sulphuric acid.
2. 2% 2,4 dinitrophenylhydrazine in 9N sulphuric acid stored at 5°C when not in use and filtered each time before use.
3. 10% metaphosphoric acid. This solution was kept under refrigeration when not in use. Fresh solution was prepared weekly.
4. 5% metaphosphoric acid.
5. 2% thiourea in 5% metaphosphoric acid.
6. 85% sulphuric acid.
7. Bromine.
8. Nitrogen. A cylinder of N_2 with facilities for saturating the gas with moisture.
9. Ascorbic acid standard: 100 mg ascorbic acid dissolved in 100 ml of 5% metaphosphoric acid. This solution contained 1 mg ascorbic acid per ml.

Procedure

This procedure describes the determination of total vitamin C like compounds (referred to as "total" vitamin C) represented by ascorbic acid, dehydro-ascorbic acid and diketogulonic acid, if present.

1. Extraction

(a) Two hundred g of orange pulp was homogenised with 100 ml of 10% HPO_3 for 2-5 minutes in a Waring Blender.

(b) 40 g of the above homogenised slurry was weighed into a 100 ml flask and diluted to 100 ml with 5% HPO_3 solution.

(c) The suspended solids were removed by centrifugation and the supernatant liquid was decanted.

2. Oxidation to Dehydroascorbic Acid

(a) To the solution from 1(c), 1-2 ml bromine was added and gently shaken until the solution appeared slightly yellow, decanted from excess bromine, and nitrogen gas saturated with water was passed through the solution until all the dissolved bromine was expelled.

(b) To a 10 ml aliquot of oxidised extract 2(a), 10 ml of 2% thiourea in 5% HPO_3 (Reagent 5) was added and mixed thoroughly.

3. Formation of Osazone

Aliquots (4 ml) of the sample dilution 2(b) were pipetted into three test tubes: one of the tubes was set aside to

serve as a blank, and to each of the remaining tubes 1.0 ml of 2% 2,4-dinitrophenylhydrazine reagent was added. All the tubes were placed in a water bath at $37 \pm 0.5^{\circ}C$ for exactly three hours and then placed in an ice bath.

4. Treatment with 85% H_2SO_4 (Formation of Soluble Pigment)

While the tubes were in the ice bath, 5 ml of 85% H_2SO_4 was added to each, followed by 1.0 ml of 2% 2,4-dinitrophenylhydrazine. The tubes were removed from the ice bath and allowed to stand for exactly 30 minutes at room temperature. The 30 minutes waiting period was critical because many interfering osazones will slowly decompose in H_2SO_4, while shorter waiting time may result in a serious over-estimation of the samples.

5. Measurement of Colour

A Unicam SP500 Spectrophotometer was used to determine the percentage transmittance in the region of 490-550 nm

6. Calibration of Spectrophotometer and Calculation

The vitamin C standard solution used was in the range 1-12 µg/ml. A calibration chart was obtained by plotting percentage transmittance against concentration of vitamin C. The total vitamin C content of each aliquot was obtained from this chart according to the formulae:

$$\text{mg total vitamin C per 100 ml of juice} = \frac{R}{W} \times \frac{100}{1000}$$

where R = mg total vitamin C per 100 ml solution obtained by reading from the calibration chart

W = g of sample per ml of the diluted solution.

RESULTS AND DISCUSSION

Table 6 and Fig. 10 show the changes in vitamin C during development and maturation.

The amount of vitamin C in August was recorded as 54 mg/100 g while by March this value was 50 mg/100 g, indicating that there was no significant change in the total vitamin C content during the development and maturation of the orange. The results obtained here are similar to those of Harding et al.(1940) working with Jaffa oranges, and Eakes (1964) working on Valencia oranges, weighing from 0.6 to 150 g.

Values in this study for total vitamin C content were slightly higher than those reported by Harding et al. (1940) working on Valencia oranges. Nagy (1980) has stated that as a general rule, those environmental conditions which increase the acidity of the fruit will also increase the vitamin C content.

Immature Valencia oranges used by Harding et al (1940) gave a maximum level of organic acids of 1.5 g/100 ml juice while the vitamin C content was maximally 38 mg/100 ml juice of mature oranges. In the present study the maximum value for total acids in immature orange juice was 2.3 g/100 ml juice (p.50) with a maximal vitamin C content of 51 mg/100 g pulp of mature oranges. This could explain the difference in values found. On the other hand, the temperature might be responsible for the different values obtained.

Table 6. Seasonal changes in the vitamin C content of Sia-Varaz Orange pulp. Graphical presentation of the data is shown in Fig. 10

Sampling time (month)	mg/100 g pulp	mg/orange pulp
Aug	54.0 n.s.	15.1 a
Sept	53.6 n.s.	27.8 ab
Oct	52.2 n.s.	25.6 abc
Nov	52.7 n.s.	38.5 cd
Dec	51.8 n.s.	53.0 e
Jan	51.1 n.s.	55.6 ef
Feb	50.6 n.s.	55.9 fg
Mar	50.0 n.s.	56.6 gh

n.s. = not significant

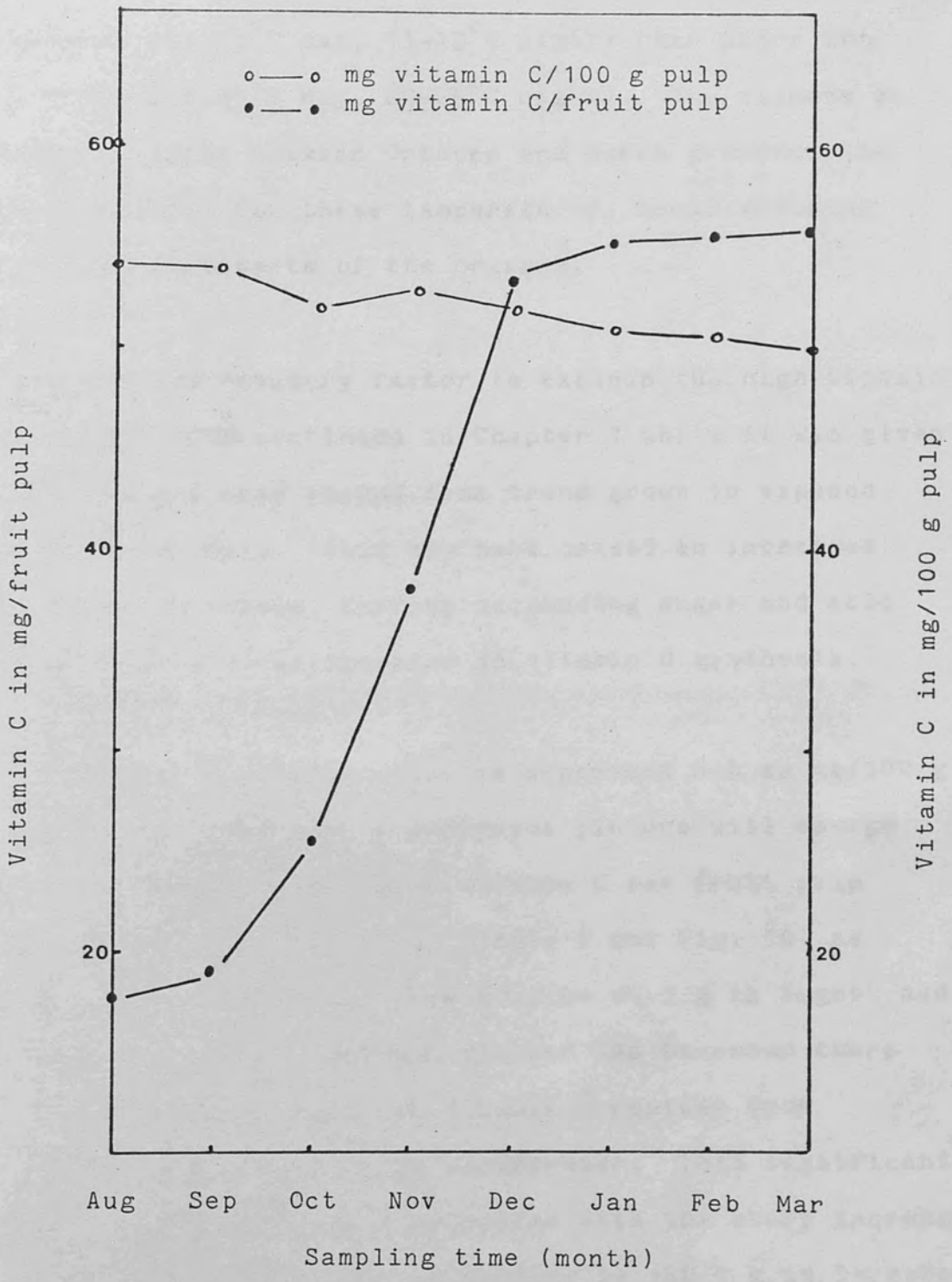


Fig. 10. Seasonal changes in the vitamin C contents of the Sia-Varaz orange fruit pulp. For significant differences refer to Table 6

It has also been shown by Rygg and Getty (1955) that fruits contained more vitamin C when grown under cool temperatures (20-22°C day, 11-13°C night) than under hot temperatures (30-35°C day, 20-25°C night). The climate in the Caspian Region between October and March provides the optimum condition for these temperatures, hence ensuring high vitamin C contents of the oranges.

Another contributory factor to explain the high vitamin C content was that mentioned in Chapter 1 where it was given that the oranges were picked from trees grown in exposed parts of the orchard. This may have caused an increased rate of photosynthesis, thereby increasing sugar and acid contents leading to an increase in vitamin C synthesis.

If vitamin C concentration is expressed not as mg/100 g pulp but as mg/fruit pulp a different picture will emerge (Table 6). The milligrams of vitamin C per fruit pulp increased from 15 to 26 mg (Table 6 and Fig. 10) as the fruit weight increased from 60.2 to 94.3 g in August and October respectively. Between October and December there was a significant increase in vitamin C content from 26 mg in October to 53 mg in December. This significant increase in vitamin content coincides with the sharp increase in fruit weight from 94.3 g in October to 160.1 g in December. From December to March the vitamin C content showed no significant change. However, the results indicated that there was an upward trend which caused the concentration of vitamin C to reach 57 mg/fruit pulp by March.

The National Academy of Sciences (Food and Nutrition Board, 1974) has recommended a daily intake of vitamin C of 35 mg (infants) to 80 mg (lactating females) whereas the Food and Drug Administration (FDA, 1973) considers an intake of 60 mg per day essential for adults and children from four years or more of age.

From the results of this study it can be seen that the daily consumption of one mature orange fruit (i.e. picked in March) provides 56.6 mg of vitamin C. This fulfils the minimum requirement set by the FDA and hence would provide enough vitamin C for good health in an adult.

CHAPTER IV

SEASONAL CHANGES IN FREE AMINO ACIDS

INTRODUCTION

Plants may utilize several chemical forms of nitrogen for growth, but only nitrogen reduced to the level of ammonia is incorporated directly into organic substances (Bryan, 1976). The largest fraction within the nitrogenous group in citrus fruits is the free amino acids, which accounts for about 70% of total soluble nitrogen in various citrus juices (Clements and Leland, 1962a). The remainder of the nitrogen is in the form of proteins, enzymes, phenolic amines, nucleotides, nucleic acids, phospholipids, vitamins and inorganic forms (Vandercook, 1977).

The carbon skeleton of the common amino acids is derived from very few metabolic intermediates, each of which is associated with a central metabolic pathway. These pathways include reactions of carbon fixation, glycolysis and tricarboxylic acid cycles.

Sims et al. (1968) have indicated that synthesis of glutamate by reductive amination of α-Ketoglutarate is the primary mechanism of ammonia assimilation in plant cells.

Because of the part played by amino acids in the storage of orange juice these have been studied extensively.

The amino acid content may determine the maturity of the fruit, may help in estimating the quality of its juice and may also help in the study of darkening of orange juice products.

Twentyfour amino acids have been reported in different varieties of orange juice using different methods of extraction and identification (Sinclair et al., 1935; Rockland et al., 1950; Underwood and Rockland, 1953; Clements and Leland, 1962a; Vandercook and Price, 1972). The role of amino acids in the development and post-harvest physiology of fruits generally has received considerable attention, as has the effect of maturity on free amino acids in orange juice. However, changes in free amino acids in the earlier stages of orange fruit development has not been so well studied. Information regarding these compounds should be of value in understanding the physiological and biochemical processes involved in fruit development, maturation and quality.

In general, results from other studies have shown significant increases in the concentration of arginine and of proline in Valencia and Washington Navel oranges during maturation. Several workers have attempted to utilize these changes as a measurement of maturity (Rockland, 1958).

Rockland and Underwood (1961) have reported changes in several amino acids of California Valencia oranges from March to July: an increase in aspartic acid, glutamic acid,

serine and arginine was observed while alanine remained relatively constant and proline increased through to May and then decreased. Ting and Deszyck (1960) have reported an increase in both total amino acids and proline from February through to May in commercial Florida orange juice.

The changes in free amino acids of several Italian orange varieties have been studied by Zamorani et al. (1972). They noted that total free amino acids increased with maturity. In most varieties studied, aspartic acid, asparagine and glutamic acid as a percentage of free amino acids decreased, while the percentage of arginine increased as the fruit was maturing.

On the other hand, Wedding and Horspool (1955) have reported wide seasonal fluctuations in the different free amino acids in citrus juice and have concluded that factors other than maturity are influencing the amino acid concentration of orange juice.

To obtain an accurate picture of what amino acids are more labile in Sia-Varaz orange juice, the effect of maturation on free amino acids was thoroughly investigated. A further point of interest was to observe changes in amino acids of the juice and to find out if amino acids could be used as markers to indicate the onset of maturity

MATERIALS AND METHODS

Extraction of Free Amino Acids

For the extraction of free amino acids from orange juice the method described by Zamorani et al. (1972) was used.

Fifty oranges were cut in half and the juice expressed by hand squeezing. Fifty ml of combined orange juice was centrifuged for 15 minutes at 5,000 r.p.m. Ten ml of the clear orange serum was transferred to a 100 ml volumetric flask and made to volume with 95% ethanol. The solution was mixed thoroughly, and stored at 4°C for 24 hours, then it was filtered through Whatman No.1 filter paper. The filter paper was washed four times with 50 ml 95% ethanol, the filtrates mixed and evaporated under reduced pressure at 40°C. The dried residue was dissolved by adding 10 ml of lithium citrate buffer solution (see below) prior to analysis on an automated amino acid analyser.

Lithium Citrate Buffer

The buffer solution was prepared by the method of Benson and Gordon (1967). Lithium citrate $4H_2O$ (14.1 g); 13.1 ml concentrated hydrochloric acid; 10 ml thiodiglycol (25% w/w in water) and 0.05 ml pentachlorophenol (see below) were added, and the volume made up to 500 ml with distilled water. The pH of this buffer was 2.2.

Pentachlorophenol solution was made by adding 50 mg of pentachlorophenol to 10 ml of 95% ethanol.

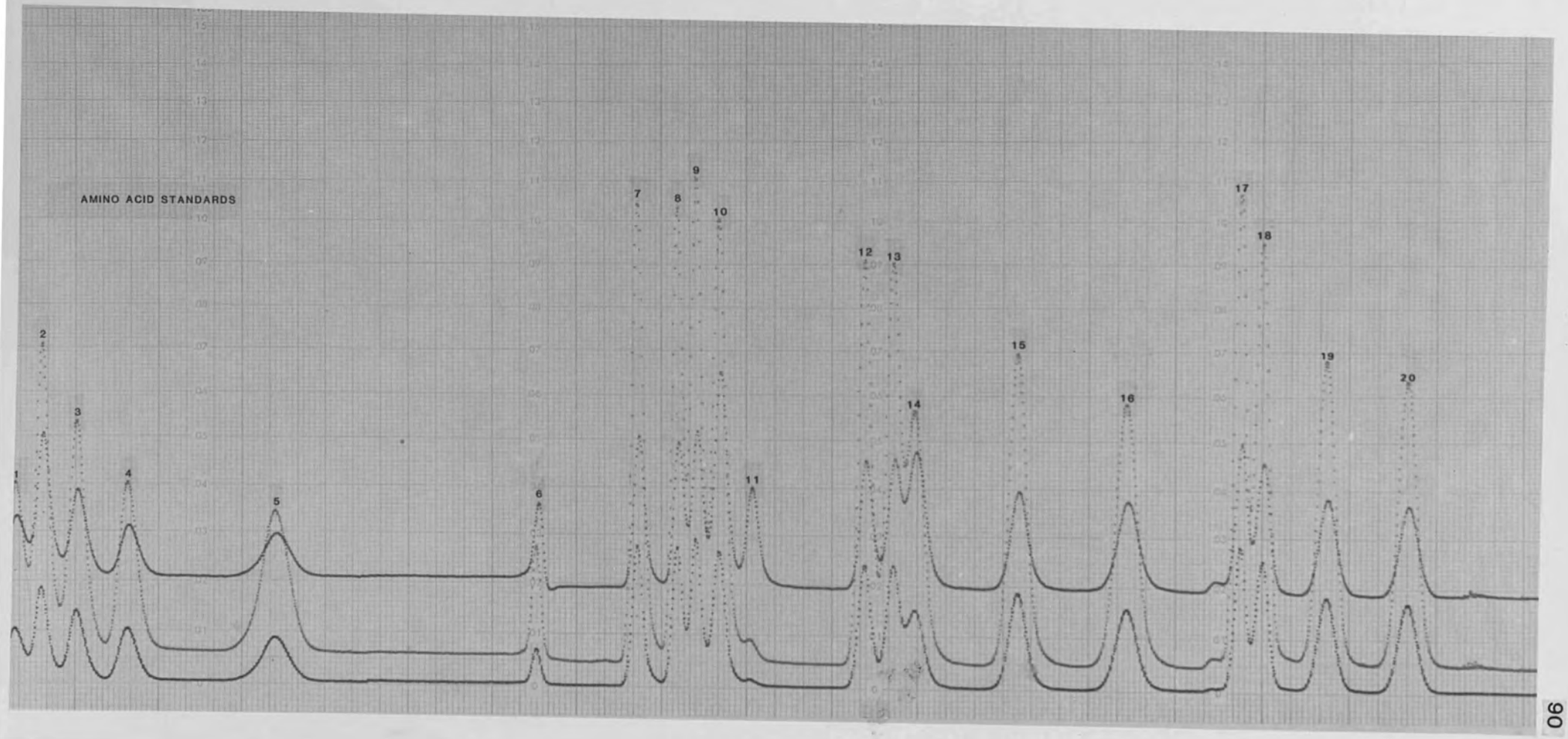
Identification and Quantification of Individual Amino Acids

The separation and quantitative determination of amino acids was carried out by using an amino acid analyser (Joel Model JLC 6AH fully automatic). In principle, this instrument automatically records the intensity of the colour produced by ninhydrin reaction with individual amino acids after their separation on ion exchange columns. The influent buffer is pumped at a constant rate through a column of sulfonated polystyrene resin. The effluent is met by a capillary Teflon tubing immersed in a boiling water bath. The absorbance of the resulting solution is measured continuously at 570 and 440 nm as it flows through a cylindrical glass cell. The peaks on the recorded curves can be integrated with a precision of 100 $\pm$ 3% for loads from 0.1 to 3.0 mMoles for each amino acid (Spackman et al., 1958).

The amount of amino acid in each sample was calculated by comparison of peak area (Fig. 13) with those obtained (Fig. 11) using a calibration mixture as described by Eveleigh and Winter (1970).

Fig. 11. Column chromatographic separation of free amino acids standards by automatic amino acid analyser

1. Tryptophan
2. Lysine
3. Histidine
4. Ammonia
5. Arginine
6. Buffer
7. Aspartic acid
8. Threonine
9. Serine
10. Glutamic acid
11. Proline
12. Glycine
13. Alanine
14. Cysteine
15. Valine
16. Methionine
17. Iso-leucine
18. Leucine
19. Tyrosine
20. Phenylalanine


AMINO ACID STANDARDS
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RESULTS AND DISCUSSION

For convenience, general grouping or families of amino acids have been delineated on the basis of their precursors. This is particularly convenient when considering branched pathways where two or more amino acids share a common precursor. Four general divisions of amino acids are indicated in Fig. 12 by the dashed lines which separate four quadrants. These include the glutamate family (A), pyruvate and serine families (B), aspartate family (C), and histidine and aromatic families (D). It should be emphasised that any such subdivision of metabolic pathways may over-simplify interactions that occur *in vivo*.

Seventeen free amino acids were identified in Persian orange juice (Fig. 13).

Glutamate family

Glutamic acid, Proline and Arginine:

One of the pathways for glutamic acid formation is the amination of α-ketoglutarate by the action of α-glutamate dehydrogenase:

$$NH_3 + \alpha\text{-ketoglutarate} + ATP + (2H) \rightleftharpoons \text{glutamate} + ADP + H_2O$$

The other pathway involves the coupled activities of glutamine and glutamate syntheses and requires glutamine as follows: (p 95)

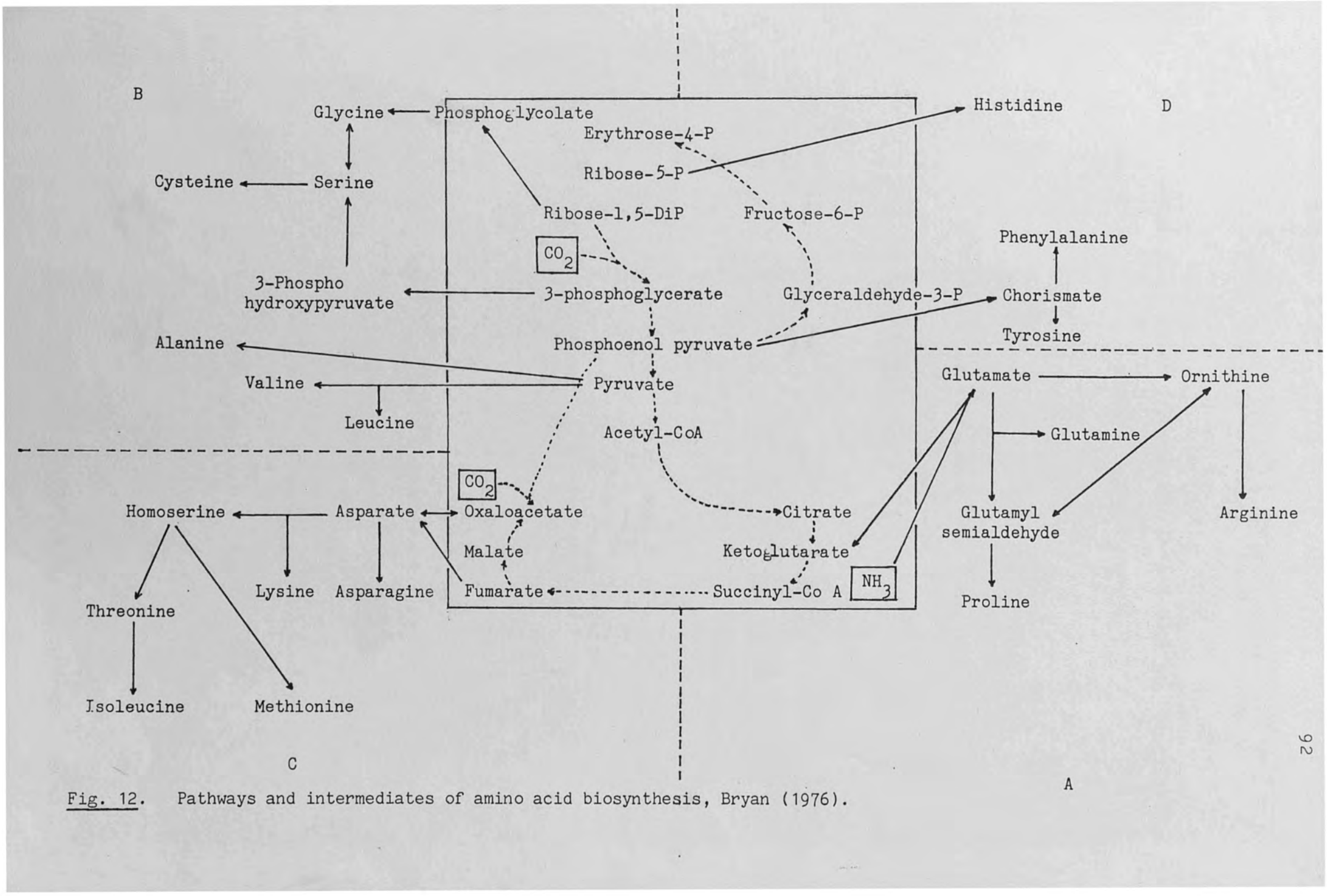
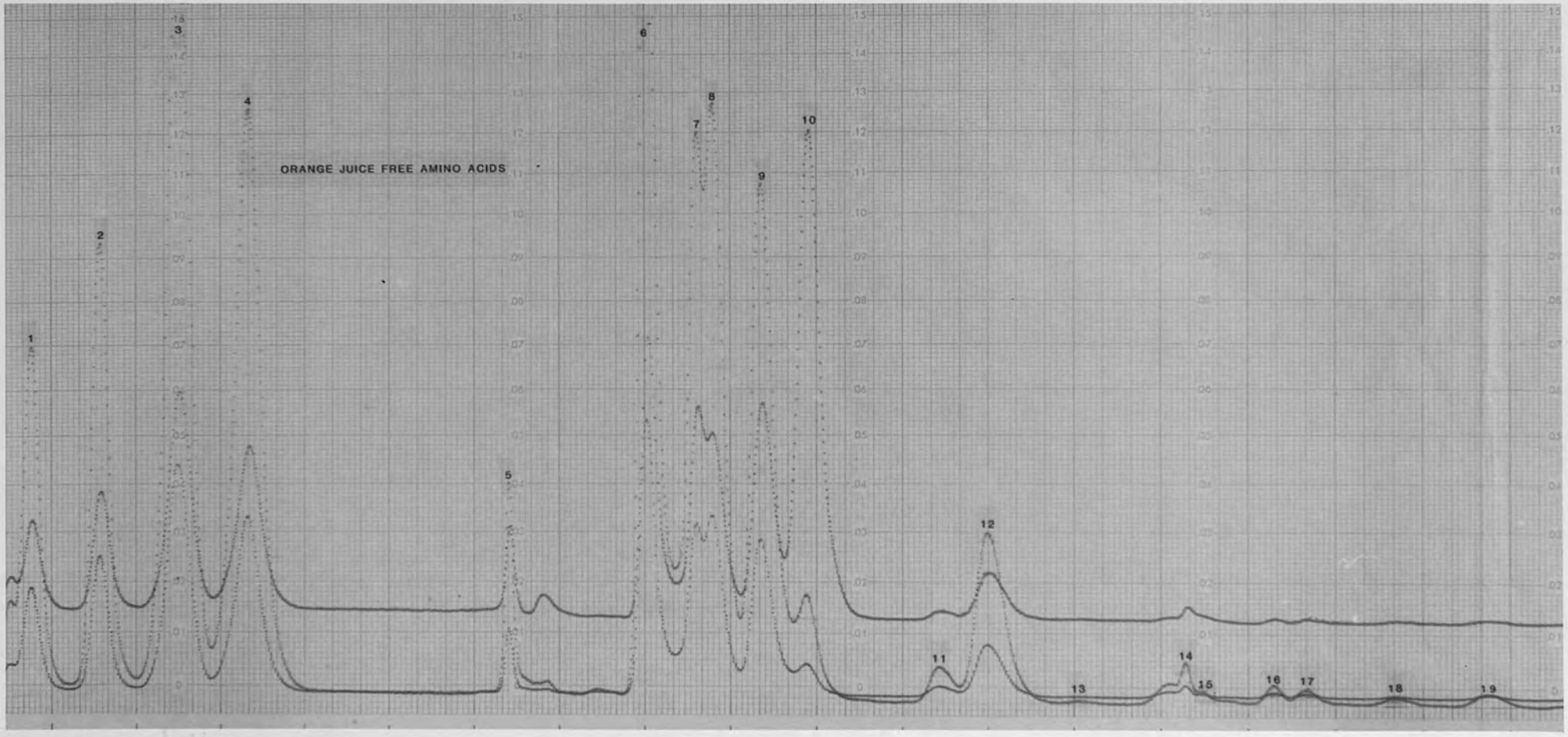


Fig. 12. Pathways and intermediates of amino acid biosynthesis, Bryan (1976).

Fig. 13. Column chromatographic separation of free amino acids of orange juice by automatic amino acid analyser

1. Lysine
2. Histidine
3. Ammonia
4. Arginine
5. Buffer
6. Aspartic acid
7. Threonine
8. Serine
9. Glutamic acid
10. Proline
11. Glycine
12. Alanine
13. Cysteine
14. Valine
15. Methionine
16. Iso-leucine
17. Leucine
18. Tyrosine
19. Phenylalanine


ORANGE JUICE FREE AMINO ACIDS
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$$\text{Glutamate} + NH_3 + ATP \rightleftharpoons \text{glutamine} + ADP + P_i + H_2O$$

$$\text{Glutamine} + \alpha\text{-ketoglutarate} \rightleftharpoons \text{2-glutamate} + (2H)$$

$$NH_3 + ATP + \alpha\text{-ketoglutarate} + (2H) \rightleftharpoons \text{glutamate} + ADP + P_i + H_2O$$

Glutamine is also required for the synthesis of several amino acids, e.g. histidine, tryptophan and arginine. On the other hand, glutamate is the precursor of proline and of arginine in plants (Bryan, 1976).

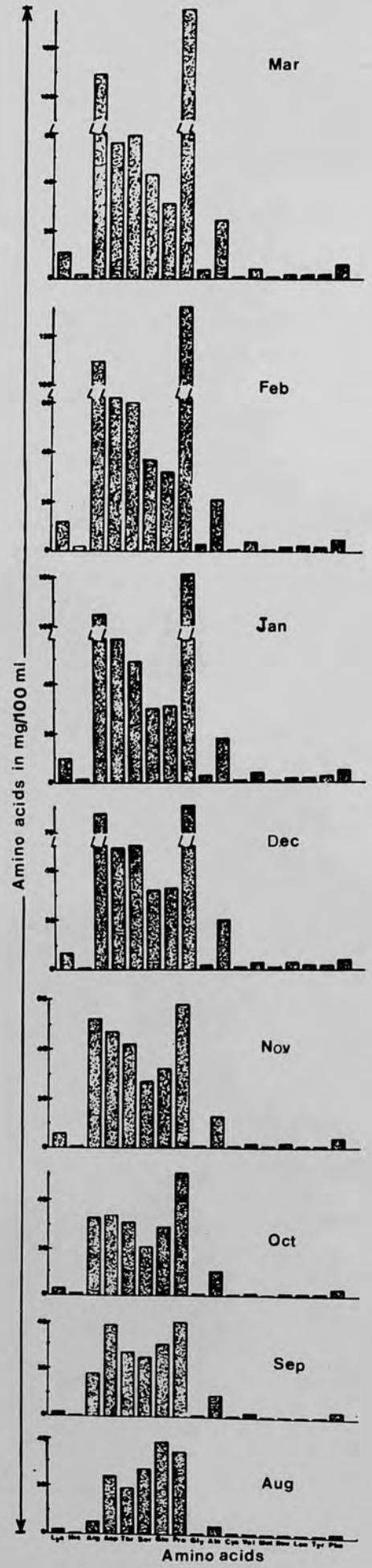
Glutamic acid has the highest value in the August samples (Table 7 and Fig. 14), and was the only amino acid showing a net decrease of 7 mg/100ml while proline, the second most abundant amino acid in August with 34 mg/100 ml, showed no significant increase in the first stage of fruit development, but increased substantially during fruit enlargement, reaching 80 mg/100 ml in December. From which was found to be highly significant. Arginine is another amino acid belonging to the glutamate family. Its concentration in August was relatively low (5 mg/100 ml). During stages 1 and 2 of fruit development, it showed a significant increase reaching 78 mg/100 ml in December. It showed a sharp increase in January reaching 105 mg/100 ml but this rate was slowing down during February and March reaching a maximum of 120 mg/100 ml in March.

Table 7. Seasonal changes in the free amino acids of the Sia-Varaz orange juice. Values are expressed as mg/100 ml. Graphical presentation of the data is shown in Fig. 14

Amino acids	Sampling time (month)							
	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar
Lys	2.4 a	2.3 ab	2.8 abc	5.7 d	7.1 de	10.1 f	12.3 fg	11.2 gh
His	Tr a	0.2 b	0.8 c	1.2 d	1.5 de	1.5 ef	2.0 fg	1.8 gh
NH_3	19.2 fgh	17.2 defg	16.3 bcde	17.1 def	15.4 bcd	12.2 a	13.7 ab	13.8 abc
Arg	5.1 a	18.1 ab	32.7 c	50.4 d	78.1 e	105.2 f	110.4 fg	119.6 gh
Asp	24.2 a	38.3 bc	33.5 ab	47.3 cd	49.6 de	58.1 fg	62.0 fgh	56.9 ef
Thr	19.1 a	27.4 ab	30.0 abc	42.4 cd	50.1 def	48.3 de	60.8 efgh	59.2 efg
Ser	27.2 bc	25.5 ab	20.2 a	27.3 bcd	32.5 cdef	30.2 bcde	37.4 fg	43.2 h
Glu	38.1 gh	30.5 ab	27.4 a	32.1 abcd	33.5 bcdefg	30.7 abc	32.4 abcdef	31.2 abcde
Pro	34.1 a	39.2 ab	50.8 abc	58.2 bcd	80.4 e	120.7 f	132.2 fg	145.2 gh
Gly	0.7 a	0.6 ab	0.8 bc	1.5 d	1.9 e	2.7 f	3.3 g	3.6 h
Ala	4.2 a	8.9 b	10.2 bc	13.1 cd	20.4 ef	17.6 e	21.6 fg	24.7 h
Cys	0.1 a	0.2 d	0.1 a	0.4 c	0.6 d	0.7 de	0.8 ef	0.9 fg
Val	1.2 a	1.8 bc	1.3 ab	2.4 cd	2.9 de	3.8 fg	3.7 def	4.3 fgh
Met	0.1 a	0.3 b	0.5 c	0.8 ef	0.6 cd	0.5 c	0.7 cde	0.6 cd
Ileu	0.2 a	0.7 b	1.3 c	1.9 d	2.6 h	2.3 g	2.0 de	2.1 def
Leu	0.1 a	0.4 b	0.9 c	1.2 d	1.8 e	2.0 f	2.4 h	2.2 g
Tyr	0.3 ab	0.2 a	0.7 c	1.1 d	1.6 e	2.5 gh	2.0 f	2.3 g
Phe	2.2 a	2.8 b	3.6 c	3.9 cd	4.2 de	5.1 fg	5.0 f	5.8 h
Total	178.0	214.6	233.9	308.0	384.8	454.2	504.7	528.6

The values obtained for cysteine through to phenylalanine are low but consistent in all replications.

Fig. 14. Seasonal changes in free amino acids of the Sia-Varaz orange juice. For significant differences refer to Table 7


Mar
Feb
Jan
Dec
Nov
Oct
Sep
Aug
Amino acids in mg/100 ml
Amino acids

Pyruvate and Serine Families

Alanine, Valine, Leucine, Serine, Glycine and Cysteine: In plant cells the principal precursor of alanine, as well as leucine and valine, is pyruvate. This keto acid acts as the amino acceptor in the direct synthesis of alanine by transamination.

The concentration of alanine was 4 mg/100 ml in August, increasing significantly during stages 1 and 2, to reach 20 mg/100 ml in December. Then there was no significant increase in the early stages of maturation but was followed by a substantial increase reaching 25 mg/100 ml in March.

Valine and leucine levels in August were very low, being 1 and 0.1 mg/100 ml respectively. During fruit development and maturation both amino acids showed a significant increase reaching 4 and 2 mg/100 ml respectively in March.

Serine, glycine and cysteine may be derived from 3-phosphoglycerate via two independent routes. One, termed the phosphorylated pathway, and the alternate pathway involves loss of phosphate during the initial formation of D-glycerate from 3-phosphoglycerate, and is therefore referred to as the non-phosphorylated pathway. Glycine can be both a product of serine metabolism and a precursor of serine synthesis.

Serine may also give rise to cysteine. In this pathway the sulphur atom of methionine is transferred to replace the hydroxyl oxygen atom of serine, thus converting serine into cysteine.

Serine was the third most abundant amino acid in August samples (27 mg/100 ml). It was constant between September and October. Its concentration increased substantially in November with no significant change between November and January. During maturation its concentration increased significantly to 37 and 43 mg/100 ml by February and March respectively.

The initial concentration of glycine and cysteine was 1 and 0.1 mg/100 ml respectively. They both showed a substantial increase during fruit development and maturation reaching 4 and 1 mg/100 ml by March respectively.

Aspartate Family

Aspartic acid, Lysine, Methionine, Threonine and Isoleucine:

Aspartic acid is synthesized by transamination of oxalacetate, with glutamate acting as the primary amino donor. A second potential mechanism of aspartic acid synthesis is the direct amination of fumarate with ammonia.

In higher plants synthesis of lysine proceeds via diaminopimelic acid. The diaminopimelic route begins with

aspartic semialdehyde and pyruvate which, after a series of reactions, including aldol condensation, dehydration and decarboxylation, yields lysine.

Threonine and methionine both have a common denominator, their four carbon skeletons arise from homoserine, a four-carbon analogue of serine. The carbon chain of homoserine is, in turn, derived from aspartic acid by a series of reactions.

The pathway to isoleucine synthesis begins with the formation of α-ketobutyric acid, which is derived from threonine.

Among the aspartate family only aspartic acid and threonine was found in comparatively high concentration in August (24 and 19 mg/100 ml respectively) while lysine, methionine and isoleucine were in small concentrations.

Aspartic acid concentration increased during stage 1 snd 2 of fruit development but remained constant during the maturation period.

Threonine showed the same pattern as that of aspartic acid with a significant increase in the first six months of fruit development, and then remaining constant for the last two months of maturation with a final concentration of 59 mg /100 ml in March.

Lysine showed a substantial increase throughout development and maturation of the fruit with its maximum concentration of 11 mg/100 ml in March.

Methionine was very low in August (0.1 mg/100 ml) increasing significantly to 0.6 mg/100 ml by March. The low concentration of cysteine during the three stages of development could be partly due to a low content of methionine during the fruit development cycle, since methionine is needed for its formation.

Isoleucine showed a substantial increase from 0.2 mg/100 ml in August to a maximum of 3 mg/100 ml in December, which then decreased to 2 mg/100 ml by March.

Histidine and Aromatic Family

Histidine, Tyrosine and Phenylalanine:
All these three amino acids showed a substantial increase during fruit development and maturation. Histidine was found in trace amounts in August but showing a continuous increase in the following months, reaching 2 mg/100 ml by March.

Tyrosine and phenylalanine was 0.3 and 2 mg/100 ml respectively in August, rising to 2 and 6 mg/100 ml respectively by March.

CONCLUSION

Table 7 presents the concentration of free amino acids during development and maturation of Sia-Varaz sweet orange juice expressed as mg/100 ml. To permit a direct comparison of the profiles, the results are shown graphically in Fig. 14.

Seventeen amino acids were identified (Fig. 13). These were lysine, histidine, arginine, aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, cysteine, valine, methionine, isoleucine, leucine, tyrosine and phenylalanine.

From the first sampling period in August to the last one in March there was a steady but significant increase in total free amino acids from 178 to 529 mg/100 ml. This average rate of increase was relatively slow during the stage of cell division (18 mg/month) but was substantially increased during cell enlargement (57 mg/month) and subsequently slowing down during maturation (48 mg/month).

The predominant amino acids in the earlier samples were aspartic acid, threonine, serine, glutamic acid and proline with arginine and alanine in appreciable amounts as well. The latter two, however, were predominant in the mature orange. Ammonia was also present in substantial amounts but showed a steady decrease during development.

This may have been due to its utilization in the synthesis of amino acids leading to the increases observed in the latter. Glutamic acid had the highest value in the immature fruits, and was the only amino acid showing a net decrease on maturation of the fruit. This decrease, however, showed an inconsistent pattern. The latter observation was also reported by Clements and Leland, (1962b); Zamorani et al., (1972) for Valencia oranges.

Both proline and arginine showed the largest increase, proline from 34 to 145 mg/100 ml corresponding to an increase of 111 mg/100 ml while arginine increased from 5 to 120 mg/100 ml corresponding to a net gain of 115 mg/100 ml. . An interesting observation was that both of these demonstrated sharp increase between December and January, coinciding with the initiation of the maturation stage in the Persian orange. This sharp increase was not noted in any of the other major amino acids. This is in direct agreement with the results of Clements and Leland (1962a, b). Apart from glutamic acid (already discussed) all other amino acids increased to larger or lesser extent.

The above observation was not in complete agreement with that of Rockland and Underwood (1961) who reported that increases were mainly in aspartic acid, glutamic acid, serine and arginine while proline increased initially but began to decrease during maturation with alanine levels remaining almost constant.

From the results of the present study it may be concluded that free amino acids that are characteristic of oranges in general were present in varying amounts, and any differences found in the Sia-Varaz variety appeared to be quantitative rather than qualitative.

The main point emerging from these results are demonstrated by the sharp increase in proline and arginine between December and January which may be related to, or may indicate, the initiation of the maturation process of Sia-Varaz orange fruits.

CHAPTER V

SEASONAL CHANGES IN THE LIPIDS

INTRODUCTION

It is well known that lipids occur in all parts of plants as they are essential components of all living cells and in some cases they may be found as food reserves. Recently it has been recognised that even low contents of lipids may play an important role in maintaining the texture, the flavour and the pigments in fruits and vegetables.

In general, the important classes of lipids in higher plants include simple lipids, phospholipids and glycolipids. The latter two groups are normally known as complex lipids.

The simple lipids found in the orange juice sacs are mainly monoglycerides (MG), diglycerides (DG), triglycerides (TG), free fatty acids (FFA), free sterols (FS), steryl esters (SE), and hydrocarbons (HC) which collectively make up 25-35% of total lipids (Nagy and Nordby, 1970).

The glycolipid fraction consists of sterol glycosides (SG), ceramide monohexosides (CMH), monogalactosyl monoglycerides (MGDG), and digalactosyl diglycerides (DGDG)

which comprise 20-30% of total lipids (Nordby and Nagy, 1971).

Phospholipids, which make up 50-60% of the total lipid content (Vandercook et al., 1970; Braddock, 1972), include phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), phosphatidyl glycerol (PG), phosphatidyl inositol (PI), phosphatidyl serine (PS), lysophosphatidyl choline (LPC), and phosphatidic acid (PA).

To a large extent the physical and chemical properties of lipids are related to the long hydrocarbon chains of fatty acids. A wide variety of fatty acids occur naturally in plants and are thought to be randomly distributed throughout the lipid classes (Hitchcock and Nichols, 1971).

The fatty acids have been classified according to the length of the hydrocarbon chain and subdivided further according to their degree of unsaturation. A simple notation used to describe fatty acids is Cn:x where 'n' represents the number of carbon atoms in the fatty acid chain while 'x' denotes the number of double bonds within the chain. Examples of these are C16:0, C18:0, C18:1, C18:2, and C18:3 which represent palmitic, stearic, oleic, linoleic, and linolenic acids respectively. Oleic acid (C18:1) possesses one double bond and hence would be termed a mono-unsaturate whereas C18:2 contains two double bonds and so would be called a poly-unsaturate.

The biochemistry of fatty acids has been reviewed extensively by Hitchcock and Nichols (1971) and the indications are that the first major fatty acid formed is palmitic acid (C16:0). This synthesis is catalysed by a group of enzymes collectively known as fatty acid synthetase. Further modifications involve 'elongase' reactions, in which 2-carbon units are added to lengthen the central 16-carbon fatty acid, and 'desaturase' reaction which introduce double bonds. Desaturation generally occurs at the C18 stage, with up to a possible three double bonds being introduced (Moore, 1982).

Nearly all the fatty acyl groups produced by the fatty acid synthesis systems are eventually incorporated into acyl lipids via fatty acid transferases, which transport the acids from their site of synthesis to the site of acyl lipid synthesis. Most of the available evidence (Hitchcock and Nichols, 1971) has indicated that L-α-glycerophosphate is the initial acceptor of acyl group at the primary site of glyceride synthesis, and that all classes of lipids (simple and complex) are drived from the diacyl-L-(α)-glycerophosphatidic acid (PA) thus formed (Fig. 15). The synthesis of lipid classes through PA and their interconversion is illustrated in Fig. 16.

Because of economical and nutritional importance of orange juice the effect of maturation on the chemical components of the edible portion (pulp) of sweet oranges

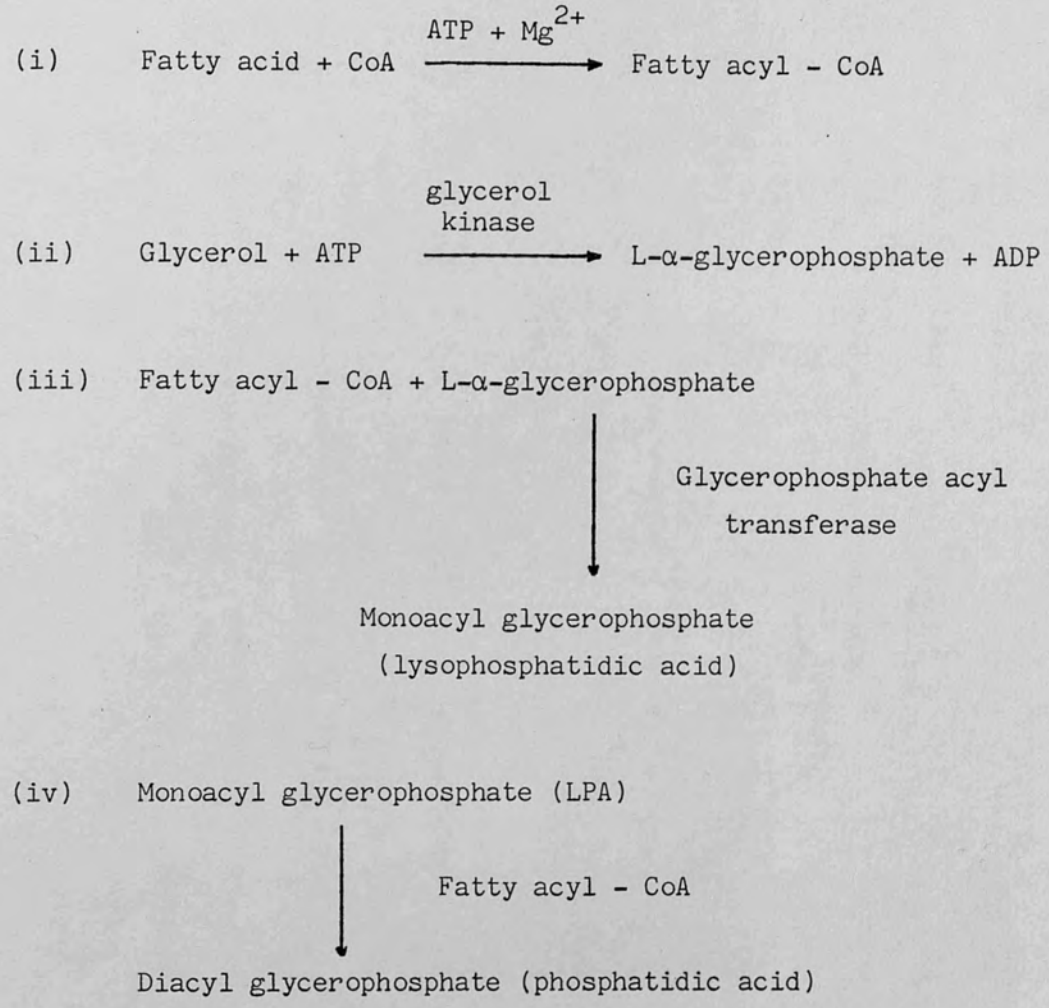


Fig. 15 Biosynthesis of phosphatidic acid (PA)

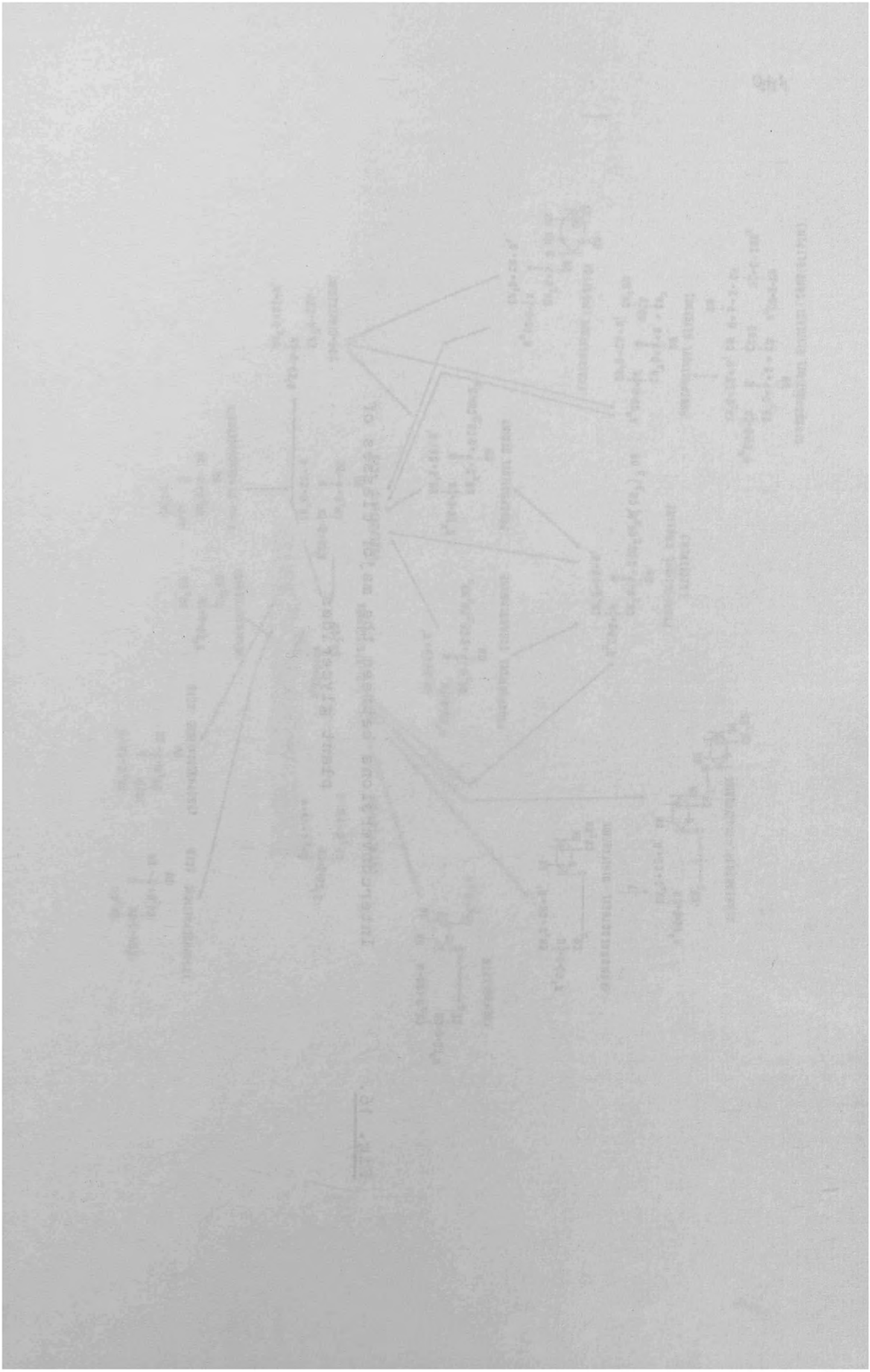
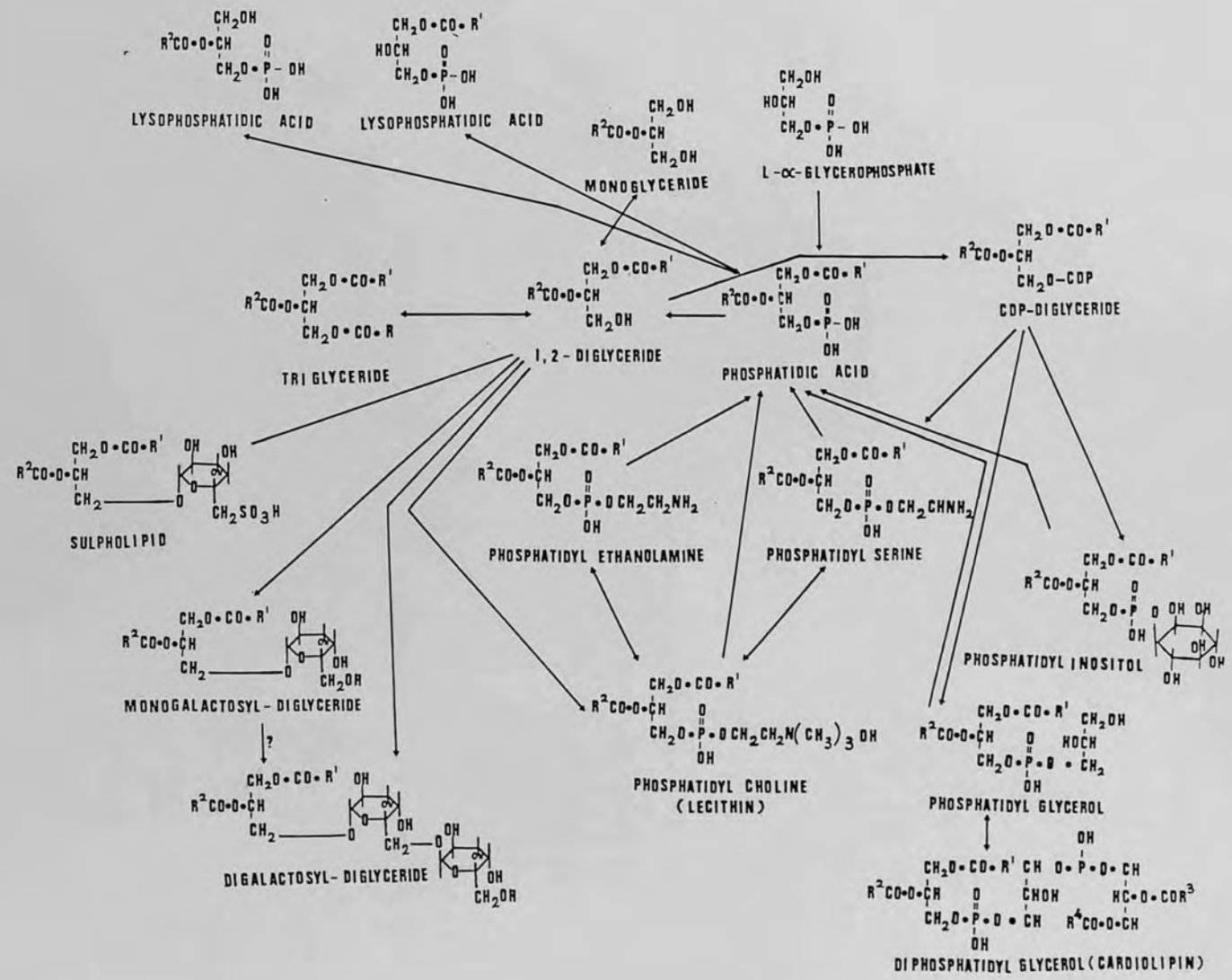


Fig. 16. Interconversions between the major classes of plant glycerides

Hitchcock & Nichols (1971)


LYSOPHOSPHATIDIC ACID
LYSOPHOSPHATIDIC ACID
MONOGLYCERIDE
L-α-GLYCEROPHOSPHATE
CDP-DIGLYCERIDE
TRI GLYCERIDE
1, 2 - DIGLYCERIDE
PHOSPHATIDIC ACID
SULPHOLIPID
PHOSPHATIDYL ETHANOLAMINE
PHOSPHATIDYL SERINE
PHOSPHATIDYL INOSITOL
MONOGALACTOSYL - DIGLYCERIDE
PHOSPHATIDYL CHOLINE
(LECITHIN)
PHOSPHATIDYL GLYCEROL
DIGALACTOSYL - DIGLYCERIDE
DIPHOSPHATIDYL GLYCEROL (CARDIOLIPIN)

has been studied the most. Some of the studies on chemical changes involved sugar content and total acid content (Harding et al., 1940; Roy, 1945), vitamin C (Atkins et al., 1945), and free amino acids (Clements and Leland, 1962a, b; Vandercook and Price, 1972), all these having been discussed in previous chapters. From these studies the typical maturation pattern which emerged was that total soluble solids and total sugar levels increased while total acids decreased. The ratio of total soluble solids to total acids has been the single most important factor for measuring maturity. However, this ratio may not be the only measure of quality, and in spite of all these studies, very little is known concerning biochemical changes in lipid composition during the development and maturation of orange fruits.

Nagy et al. (1978) have studied the changes in lipid classes of juice during the maturation of sweet oranges. Their data showed that of the three lipid fractions, only the phospholipid phosphorus fraction declined consistently after the fruit had reached maturity. Glycolipids and simple lipids showed no significant changes during that time. In a more recent study on the fatty acid composition of three varieties of sweet orange juice Nordby and Nagy (1979) have observed a close relationship between the fatty acid profile and stages of maturity.

Another parameter worth studying was therefore the lipids of the juice of Sia-Varaz oranges. This will be discussed on the following pages.

MATERIALS AND METHODS

The term LIPID is often used loosely to cover a wide variety of substances which:

are insoluble in water,

are soluble in organic solvents such as chloroform, ether, hexane, benzene and methanol,

contain long-chain hydrocarbon groups in molecules,

are present in, or derived from, living organisms.

This definition covers a wide range of compounds including fatty acids and their derivatives, steroids, terpenes, carotenoids, wax esters, aldehydes, 'fat-soluble' vitamins and bile acids, which all have in common a ready solubility in organic solvents.

Christie (1973) has used a more specific definition and the term is nowadays generally restricted to fatty acids and their derivatives or metabolites. It is in this sense that the term is used in this study.

The principal lipid classes consist of fatty acids moieties linked by an ester bond to an alcohol, principally the trihydric alcohol glycerol, or by amide bonds to long-chain bases. Also, they may contain phosphoric acid, organic bases, sugars and more complex components that can be liberated by various hydrolytic procedures. Lipids may be subdivided into two broad classes - SIMPLE, which contain one or two of these hydrolysis products per mole,

and COMPLEX, which contain three or more types of hydrolysis product per mole. The terms NEUTRAL and POLAR respectively are used more frequently to define these classes, but are less precise, and may occasionally lead to confusion, for example, unesterified fatty acids (free fatty acids) are normally classed as neutral lipids despite the presence of the carboxyl groups in their molecules.

The Isolation of Lipids from Tissues

To extract lipids from tissues the following considerations should be taken into account:

1. The solvent mixture should be sufficiently polar to remove all lipids from their association with cell membranes, and should not be so polar that triglycerides and other non-polar simple lipids are not dissolved and are left adhering to the tissue.

2. The chemicals used should not react chemically with these lipids.

Taking these considerations into account, there are many different solvent systems suggested by different authors for extracting lipids from tissues. It is generally agreed that the mixture of chloroform and methanol in the ratio of 2:1 (V/V) will extract more lipids from animal and plant tissues than most other simple solvent systems. However, the extractability of

a tissue under investigation is variable and depends on the nature of the tissue and of the lipids. Solvent systems containing a little water are sometimes recommended for extracting lipids from very difficult materials such as cereals or lyophilised orange juice and orange juice sacs.

Removal of Non-Lipid Contaminants

Most polar organic solvents used to extract lipids from tissues also extract significant amounts of non-lipid contaminants such as sugars, urea, amino acids and salts.

Most of the contaminating compounds can be removed from chloroform - methanol (2:1, V/V) mixtures simply by shaking the combined solvents with one quarter their total volume of water, or better still, with a dilute salt solution (0.88% potassium chloride solution) (Christie, 1973).

Taking all these considerations into account the method devised by Nagy and Nordby (1970) was used for extracting lipids from lyophilised orange juice sacs in the present study.

Free Lipids = Lipids

Because of the difficulty of extracting bond lipids from biological membranes, only free lipids have been extracted in the present study using Nagy and Nordby's (1970) method. So, in the following chapters the term lipids refers to free lipids.

EXPERIMENTAL

Orange fruits were carefully peeled to avoid contamination from lipids and waxes contained in the peel. The membrane was removed by means of a surgical knife. The seeds were then separated from the juice sacs. The juice sacs were transferred to a 250 ml round-bottomed flask. Freeze drying was carried out by immersing the flask into an acetone-solid carbon dioxide mixture until the contents became frozen. The flask was connected to a vacuum-dryer and left to dry for 48 hours. The powder had a moisture content no greater than 4% (Nagy and Nordby, 1970).

Total lipids were extracted from 15 g orange powder samples in the following manner:

The sample was placed in a Waring Blender and 25 ml water added for rehydration. Three hundred ml of chloroform-methanol (2:1, V/V) was added next and the total mixture blended at room temperature for 10 minutes at low speed. The mixture was filtered on a coarse sintered glass funnel and the residue subjected to two more successive 150 ml extractions with chloroform-methanol (2:1, V/V). The mixtures were filtered and the combined filtrate concentrated (*in vacuo*, at 30°C) until a thick syrup resulted. The syrup was transferred to a separating funnel with 50 ml chloroform and 50 ml water added alternately. Ammonium sulphate (ca. 1 g) was added

to the funnel to enchance separation of the biphasic system, and the contents shaken vigorously. Upon removal of the chloroform layer, the aqueous layer was extracted with 4 x 50 ml chloroform until free of chloroform-soluble pigments. The combined chloroform phases were reduced to dryness (*in vacuo*, at 30°C) and the crude lipid taken up with a known amount of chloroform.

Calculation of Total Lipids

Having obtained the lipid extract as described, it was diluted to known volume with chloroform. One ml of this solution was put in a tared vial, and the solvent was evaporated in a stream of nitrogen at 30°C; the residue was placed over fresh potassium hydroxide pellets in a desiccator and evacuated on a high vacuum pump to a pressure of 0.1 mm Hg. The sample was kept in the desiccator *in vacuo* until the weight was constant to within $\pm$ 0.05 mg (Kates, 1972). The lipid content of the sample was calculated as follows:

$$\text{total lipids} = \frac{V_1 \times W \times 100}{V_2 \times 15}$$

V_1 = Total extraction volume

V_2 = Volume of sample aliquot

W = Weight of lipid in sample aliquot

Results are expressed as per 100 g powder.

Separation of Simple Lipids into different Fractions

To separate the different fractions of simple lipids, the one-dimensional, two-step TLC procedure as described by Biezenski et al. (1968) was used. A thin-layer plate (20 x 20 cm) plastic backed, coated with a layer of 0.25 mm Silica gel G, type Polygram Sil G, supplied by Macherey-Nagel & Co. Ltd., was ruled in 10 mm wide lanes with a space of 0.5 cm between them. After applying the samples, the plate was first developed in ethyl ether-benzene-ethanol-acetic acid (40:50:2:0.2, V/V) to a distance of 10 cm above the starting line. After this first development, the plate was dried in a vacuum desiccator for 20 minutes. The plate was then placed in a solvent of hexane-benzene-acetic acid (80:20:1, V/V) and developed to a point 16 cm above the origin. The plate was removed from the chromatography tank and vacuum dried in a desiccator to remove traces of solvents (Fig. 17).

One-dimensional, two-step TLC Procedure for the Separation of Complex Lipids

Single dimensional TLC was necessary for the rapid qualitative and quantitative estimation of complex lipids.

To separate the complex lipids into individual fractions, two solvent systems were used. The first solvent system was petroleum ether (40-60^{o}C) - acetone (3:1, V/V). The unknown lipid samples from the juice sacs were applied on a plate alongside the standards, as well as together with them.

Fig. 17. One-dimension two-step separation of simple lipids of orange juice sacs on silica gel G layers

A - G are samples as follows:

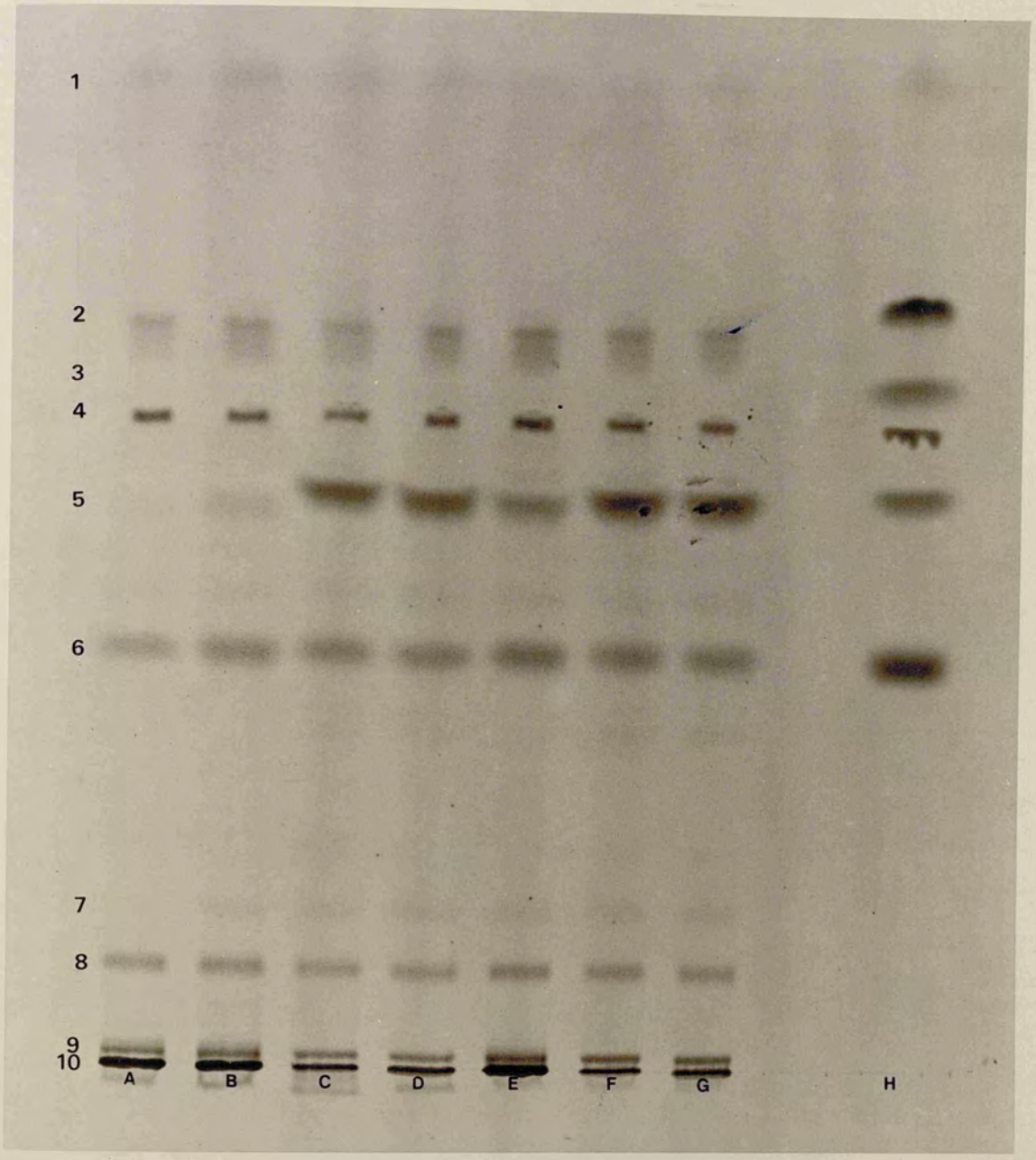
A, August; B, September; C, October; D, November;

E, December; F, January; G, February; H, Standards.

1. Hydrocarbons
2. Esterified sterols
3. Esterified fatty acids
4. Triglycerides
5. Free fatty acids
6. Free sterols
7. Unknown
8. Diglycerides
9. Monoglycerides
10. Origin

Solvent I: ethyl ether-benzene-ethanol-acetic acid (40:50:2:0.2, V/V)

Solvent II: hexane-benzene-acetic acid (80:20:1, V/V)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The plate was developed to a distance of 19 cm above the origin, removed from the tank and dried in a vacuum desiccator for ca. 20 minutes. It was then placed in a tank containing the second solvent system of chloroform-methanol-acetic acid-water (170:25:25:6, V/V) and developed up to 2 cm below the previous solvent front. The plate was then vacuum dried in a desiccator to remove traces of solvent (Nichols, 1964) (Fig. 18).

Two-dimensional TLC for the Separation of Complex Lipids

The thin-layer plate for two-dimensional chromatography was first developed to a height of 17 cm with chloroform-methanol-7N ammonia (70:30:1, V/V). The plate was removed from the tank, vacuum dried for ca. 20 minutes, turned 90° anticlockwise and placed in a chromatography tank containing chloroform-acetone-methanol-acetic acid-water (125:50:12:10:5, V/V) for development in the second direction. The plate was then vacuum dried to remove traces of the solvents (Christie, 1973) (Fig. 19).

Detection and Identification of Lipid Components

Detection of unknown lipid components on developed thin-layer plates were carried out by destructive or non-destructive, specific or non-specific reagents. They were identified by their developed colour or migration characteristics relative to authentic standards that were chromatographed alongside the unknown lipid mixtures on the same plate.

Fig. 18. One-dimension two-step separation of complex lipids of orange juice sacs on silica gel G layers

A, C, E, and G are samples

B, D, F, H, I, J, and K are standards

1. Simple lipids
2. Ceramide monohexosides
3. Monogalactosyl diglycerides
4. Unknown
5. Phosphatidic acid
6. Phosphatidyl diglycerol
7. Phosphatidyl ethanolamine
8. Unknown
9. Phosphatidyl serine
10. Phosphatidyl choline
11. Lysophosphatidyl choline
12. Phosphatidyl inositol

Solvent I: petroleum ether (40-60°C) - acetone (3:1, V/V)

Solvent II: chloroform-methanol-acetic acid-water (170:25:25:6, V/V)

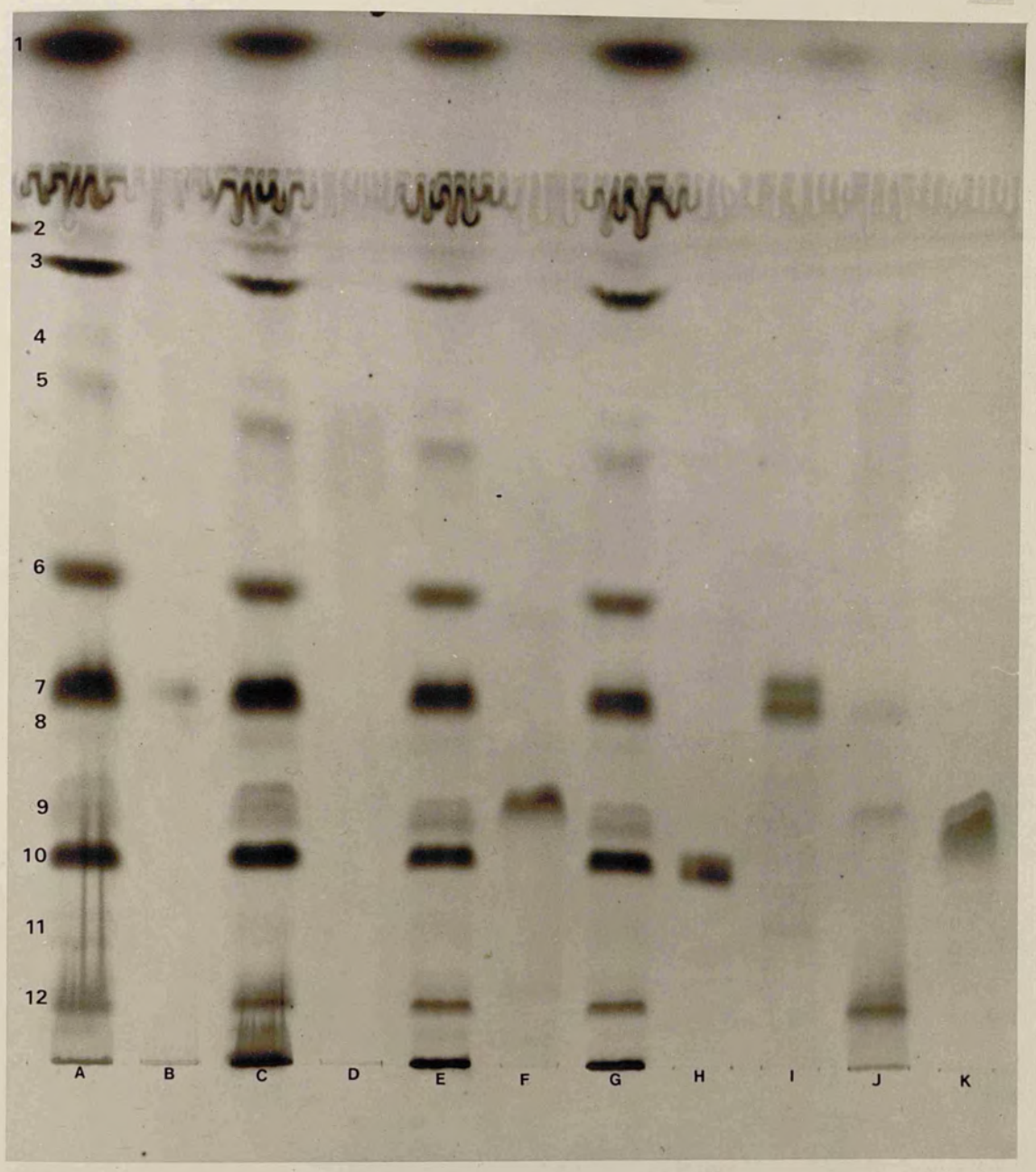
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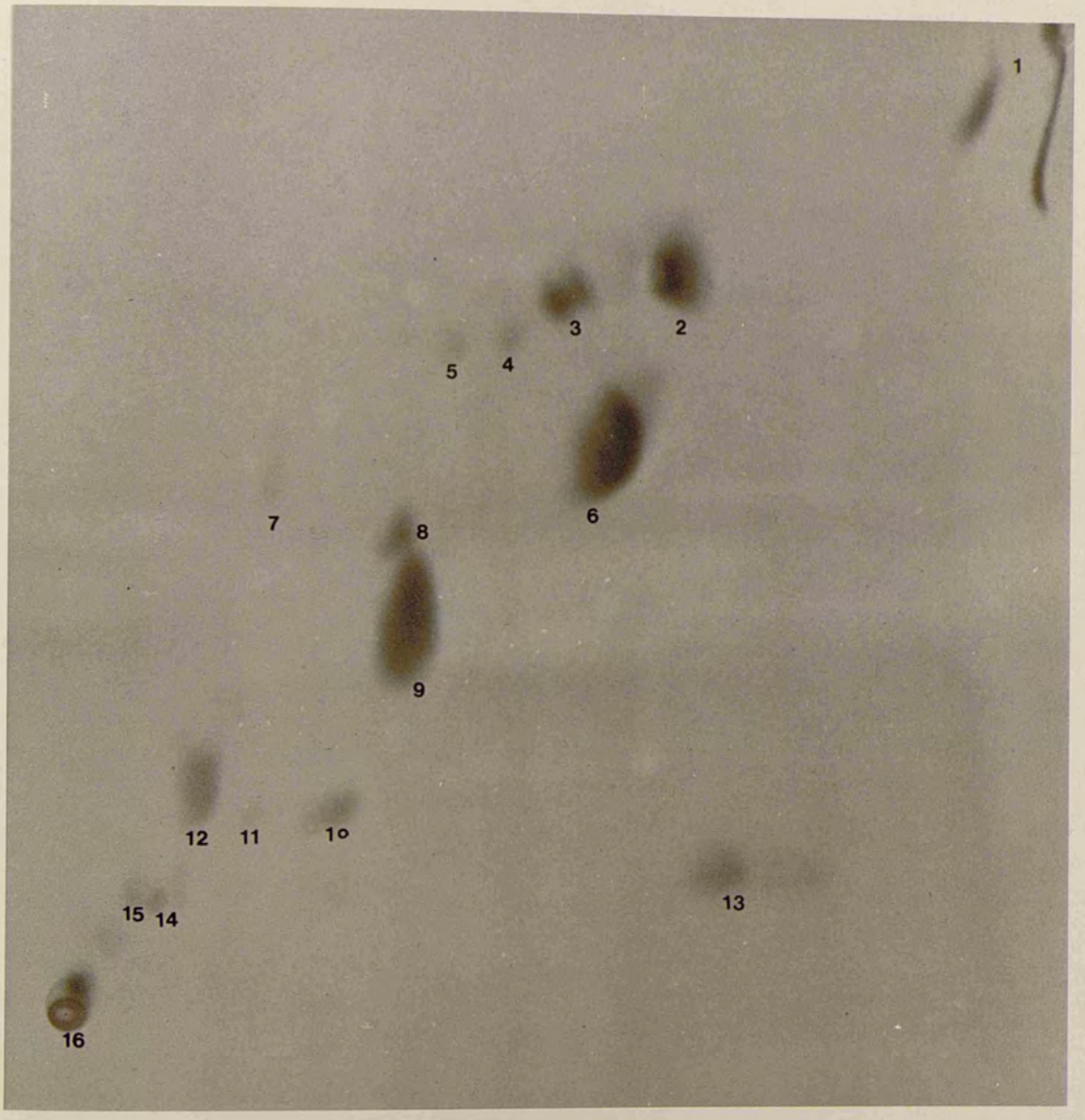
Fig. 19. Two-dimensional TLC of complex lipids of orange juice sacs on silica gel G layers

1. Simple lipids
2. Monogalactosyl diglycerides
3. Ceramide monohexosides
4. Unknown
5. Unknown
6. Phosphatidyl ethanolamine
7. Phosphatidyl glycerol
8. Digalactosyl diglyceride ?
9. Phosphatidyl choline
10. Phosphatidyl serine
11. Unknown
12. Phosphatidyl inositol
13. Phosphatidic acid
14. Lysophosphatidyl choline
15. Unknown
16. Origin

Solvent I: chloroform-methanol-7N ammonia (70:30:1, V/V)

Solvent II: chloroform-acetone-methanol-acetic acid-water (125:50:12:10:5, V/V)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A. General Detection Tests

1. Cupric acetate

A solution of 3% cupric acetate in 8% phosphoric acid was prepared and the developed thin-layer plate was sprayed with this solution and then heated at 180°C for 10 minutes. All saturated and unsaturated lipid compounds appeared as charred spots of different intensity according to their concentrations (Privett et al., 1977) (Figs. 17-19).

2. 2', 7'-Dichlorofluorescein

The developed plate was sprayed with 0.1% (W/V) solution of 2',7'-dichlorofluorescein in 95% methanol. Most lipids showed up immediately as yellow spots under UV-light. This reagent is non-destructive and is useful when the lipid fractions on the developed plate are required for further analyses (Christie, 1973).

3. Iodine vapour

The plate was hung in a development tank containing a few crystals of iodine. After a few minutes, most lipids appeared as brown spots on a pale yellow background. Iodine vapour was used to distinguish between glycolipids and phospholipids as only the latter are stained significantly under these conditions (Skipski and Barclay, 1969).

B. Specific Reagents

1. Sterols and their esters

Fifty mg ferric chloride ($FeCl_3$ $6H_2O$) was dissolved in 90 ml of distilled water with 5 ml acetic acid(glacial) and 5 ml concentrated sulphuric acid added. The developed plate was sprayed with the reagent, then heated at 100°C for 10 minutes when the presence of sterols and their esters was indicated by the appearance of a red-violet colour. The colour for sterols appeared slightly before that of their esters (Christie, 1973).

2. Free fatty acids

The developed plate was sprayed in turn with a 0.1% (W/V) solution of 2',7'-dichlorofluorescein in 95% methanol, a solution of 1% aluminium chloride in ethanol and finally with 1% aqueous ferric chloride, warming the plate briefly after each spray. Free fatty acids gave a rose-violet colouration (Christie, 1973).

3. Esterified fatty acids

Two reagents were necessary. (a) Hydroxylamine hydrochloride (10 g) was dissolved in water (25 ml) and ethanol (100 ml) and was added to a saturated aqueous solution of sodium hydroxide (26 ml) diluted with 200 ml ethanol. The mixture was filtered before use to remove any precipitated sodium chloride. (b) Ferric chloride

(10 g) and concentrated hydrochloric acid (20 ml) were ground together in a mortar, and were shaken with 300 ml diethyl ether. The developed plate was sprayed with the first reagent, dried, then sprayed with the second reagent, when esterified fatty acids appear as purple spots on a yellow background (Skipski and Barclay, 1969).

4. Phospholipids

A spray reagent specific for phosphate esters on developed thin-layer plates was particularly useful in the identification of phospholipids. A modification of the molybdenum blue reagent of Zinzadze (1935) as described by Dittmer and Lester (1964) was used to identify phospholipids.

Solution I: To 1 l of 25 N H_2SO_4, 40.11 g of MoO_3 was added and the mixture was boiled gently in a fume cupboard until the MoO_3 dissolved.

Solution II: To 500 ml solution I, 1.78 g of powdered molybdenum was added and the mixture was boiled gently for 15 minutes. The solution was cooled and decanted from any residue that may be present.

Solution III: Equal volumes of solutions I and II were mixed and the combined solution was mixed with two volumes of water. The final solution was greenish yellow in colour.

Procedure

The developed plate was sprayed lightly until the absorbent was uniformly damp. Components containing phosphate ester showed up immediately as blue spots on a white or light blue-grey background. The intensity of the colour increased on standing. After several hours the background darkened to a deep blue and the spots were obscured; therefore, plates could not be kept as a permanent record.

5. Phospholipids containing free amino end groups

Phospholipids such as phosphatidyl ethanolamine, phosphatidyl serine and related lyso compounds that have free amino groups, can be detected with the aid of a ninhydrin spray. The developed plate was sprayed with a solution of 0.25% ninhydrin in acetone, diluted with an equal volume of water immediately before use. Lipid fractions having free amino groups appeared red-violet when the plate was heated in an oven at 100^{o}C for 10 minutes (Dittmer and Lester, 1964).

6. Phospholipids containing choline

Phosphatidyl choline and lysophosphatidyl choline gave a positive reaction with the following reagent:

Reagent I: 1.7 g basic bismuth nitrate was dissolved in 100 ml of acetic acid.

Reagent II: 40 g of potassium iodide was dissolved in 100 ml water.

Reagent III: 20 ml reagent I was mixed with 5 ml reagent II and 70 ml water just before use.

Procedure

After the plate was sprayed with reagent III the choline-containing phospholipids appeared as orange to orange-red spots immediately on or after warming at 40°C for 5-10 minutes (Wagner et al., 1961).

7. Sphingolipids

Cerebrosides gave positive reactions with the Clorox-benzidine spray.

Reagent I: 50 ml benzene was mixed with 5 ml Clorox bleach (trade name of commercial bleach

active reagent sodium hypochlorite) and 5 ml of glacial acetic acid.

Reagent II: 200 ml of benzidine dihydrochloride and a small crystal of potassium iodide were dissolved in 50 ml of 50% ethanol and filtered.

Procedure

The plate was sprayed immediately with freshly-prepared reagent I, left at room temperature for 30 minutes, dried in hot air (this was done in the fume cupboard) for 10 minutes and then sprayed with reagent II (this reagent was used within two hours of preparation and protected from direct light). Cerebrosides appeared as blue spots almost immediately (Skipski and Barclay, 1969).

8. Glycolipids

As well as the rather negative use of iodine in conjunction with non-specific reagents to detect glycolipids, a number of more positive highly-specific reagents are available for the detection of the carbohydrate moieties. The reagents most widely used are (i) an orcinol sulphuric acid mixture, and (ii)α-naphthol.

(i) Orcinol spray

The reagent was prepared by dissolving 200 g orcinol in 100 ml of 75% sulphuric acid. The whole surface of the developed plate was wetted by spraying and then heated in an oven at 100°C for 10 minutes. Glycolipids appeared as blue-violet spots on a white background. The solution is stable for about one week if refrigerated and kept in the dark (Svennerholm, 1956).

(ii) α-Naphthol

Half a gram α-naphthol was dissolved in methanol-water (1:1, V/V) and sprayed on the developed plate until the surface was wet. After air drying at room temperature, the plate was sprayed lightly with 95% sulphuric acid, then heated at 120°C in an oven when glycolipids appeared as purple-blue spots and other complex lipids as yellow spots (Siakotos and Rouser, 1965).

Quantification of Lipid Components

Simple lipid and complex lipid fractions were quantified by densitometry after they were separated on thin-layer plates, sprayed with cupric acetate reagent and charred in an oven at 180°C for 10 minutes. Each series of zones were cut out and scanned in a chromoscan double beam recording and integrating densitometer (Joyce Loebl and Co. Ltd.) with quartz iodine light source, blue filter and 20 cm x 1 cm light slit and sample-holder

(drive in gear ratio was 1:1). The area of the peaks on the recorder trace is proportional to the amount of lipids originally present in the sample (Christie, 1973) and in authentic standards. Individual bands were estimated using the following formula:

$$\frac{\text{Peak area of standard}}{\text{Peak area of sample}} = \frac{\text{Amount of standard}}{\text{Amount of lipid in sample}}$$

Gas-liquid Chromatography (GLC) of Fatty Acids

Gas-liquid chromatography, a technique first introduced by James & Martin (1956), is the method of choice for rapid, quantitative analysis of volatile lipid components such as fatty acid esters, fatty alcohols, hydrocarbons, etc. In this procedure the fatty acids are first converted into a more volatile form like methyl esters. An inert carrier gas such as nitrogen is used as the moving phase for partition chromatography of the vaporized mixture of methyl esters between the moving gas phase and the stationary liquid phase of a high-melting polyester or silicone polymer coated on particles of diatomaceous earth. The methyl esters of the various fatty acids partition themselves between the moving gas phase and stationary liquid phase according to their individual gas-liquid partition coefficients. The separated methyl esters in the gas phase leaving the column can be measured by the flame-ionization detector. The carrier gas stream containing the fatty acid esters is mixed with a stream of hydrogen and air and is burnt in a high-voltage

electric field. The current generated by the flow of ionized fragments of the fatty acid in the flame is automatically recorded on a chart which shows a series of separate peaks. Each peak corresponds to a separate fatty acid, and the area under the peak is proportional to the amount present.

Fatty Acid Methyl Ester Preparation

Methyl esters of total and free fatty acids were prepared by transferring 1 ml of dissolved lipid sample (P. 118) to a 50 ml round-bottomed flask and drying under reduced pressure at 40°C. The dry lipid was refluxed for 5 minutes with 4 ml 1 N NaOH in methanol (alkaline reflux being eliminated if determination of free fatty acids was desired). After refluxing, 5 ml of 14% BF_3-methanol reagent was added and refluxing continued for an additional 5 minutes (Metcalfe et al., 1966). The esters were extracted with 2 ml of heptane, the heptane layer washed with water saturated with NaCl (Van Wijngaarden, 1967), and finally the heptane layer was dried over $Na_2 SO_4$. All esters were stored in 0.1 ml heptane at 5°C for GLC analysis.

Gas-liquid Chromatography

Fatty acid methyl esters were determined with a Pye 'series 104' gas chromatograph equipped with a hydrogen flame detector. Glass column (1.5 m in length and 2 mm in diameter) was packed with 10% polyethylene glycol-

adipate (PEG-A) on chromosorb W.AN-DMCS, 60-80 mesh supplied by Phase Separation Ltd., England. The gas chromatograph was operated with H_2 pressure of 12 p.s.i. and air pressure of 10 p.s.i., with chart speed of 76.2 cm h^{-1} on a Speedomax W recorder (Leeds and Northrup Co., England).

Two temperature conditions were investigated:

(a) temperature programmed from 80°C to 190°C at 8 min^{-1};

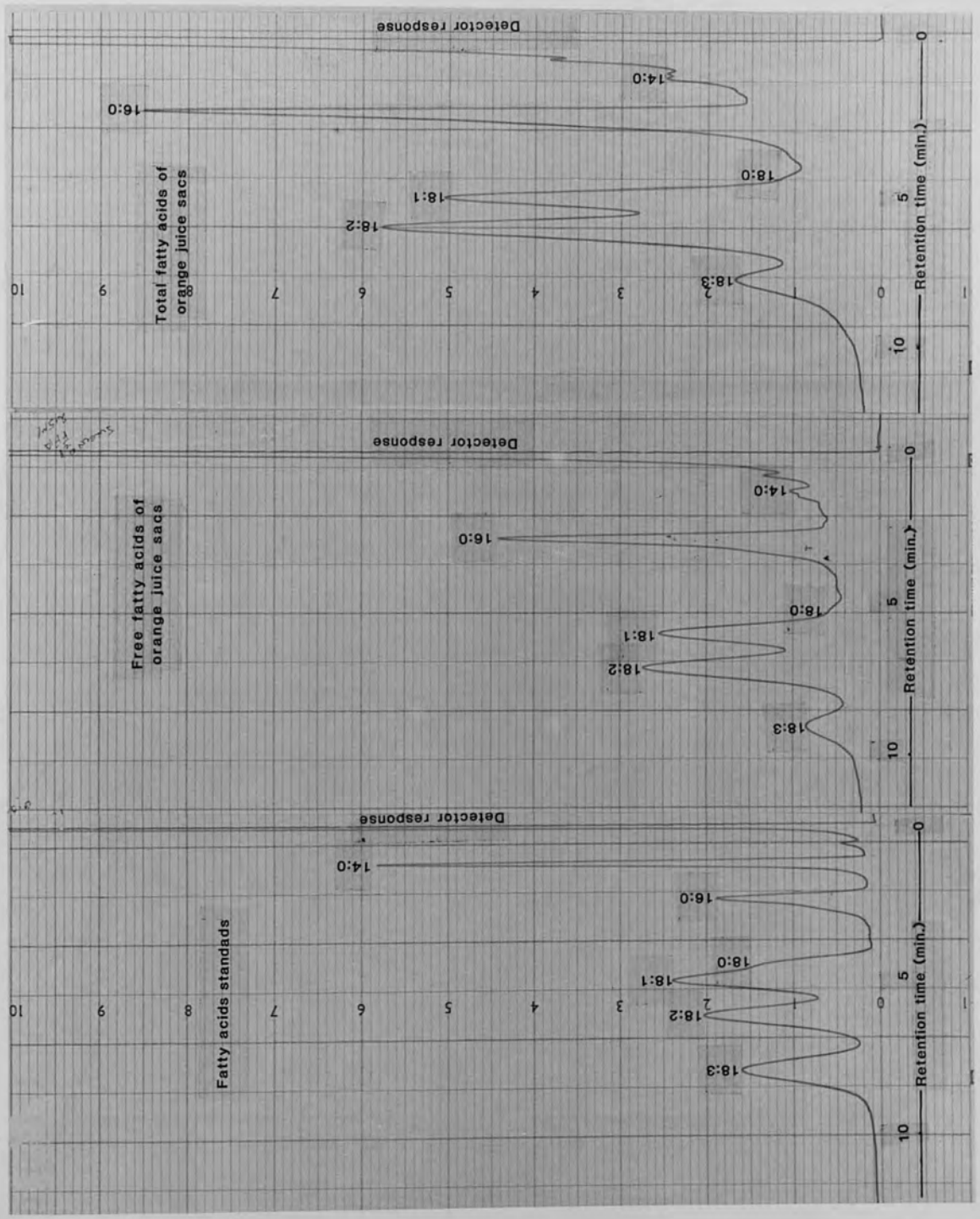
(b) isothermally at 190°C.

No differences were observed using those two temperature conditions and all work was therefore carried out isothermally at 190°C as it was the faster of the two. Fig. 20 shows the GLC of fatty acids methyl esters.

Identification and Quantification

Standard mixtures of known fatty acid methyl esters obtained from Applied Science Laboratories, England, were used for comparison of retention times, identification and the quantification of fatty acid methyl esters in each sample. In every case unsaturated acids emerged later than the corresponding saturated ones, and the degree of retention depended on the number of double bonds present (Nichols et al., 1966). The amount of each fatty acid ester was calculated as follows:

Fig. 20. The separation of fatty acid methyl esters by gas-liquid chromatography on a 150 cm x PEG-A column at 190°C


Fatty acids standards
Free fatty acids of orange juice sacs
Total fatty acids of orange juice sacs
Detector response
Retention time (min.)
14:0
16:0
18:0
18:1
18:2
18:3

$$\frac{\text{Peak area of standard}}{\text{Peak area of sample}} = \frac{\text{Amount of fatty acid in standard}}{\text{Amount of fatty acid in sample}}$$

Other calculations used were:

relative concentration of each fatty acid which was calculated by triangulation of the peak area on the chromatograms and was expressed as percentage of the total peak area (Dogras et al., 1977):

$$\text{Percentage peak i} = \frac{\text{Peak area i}}{\text{Peak area}} \times 100$$

RESULTS AND DISCUSSION

Total Lipids of Juice Sacs

The results of total lipids in juice sacs during fruit development and maturation is shown in Table 8. Total extracted lipids from juice sacs showed a continuous decrease from August to February, then it increased in March.

In August total lipids were 1726 mg/100g powder which decreased significantly to 1404 mg/100 g powder in February, then increased to 1436 mg/100 g powder in March.

The high amount of lipid on a per 100 g basis in immature juice sacs and its lower value on maturation was partly dueto the fact that the rate of sugar synthesis is very high as the orange matures (Table 5) (p.71). In the early stages of development (August and September) the amount of lipid extracted was high as it was required for cell membrane formation, at a time when the sugar content was low. As sugar accumulates, the amount of lipid extracted from the same weight of powder decreases.

Simple Lipids

Total simple lipids showed no significant change between August and October with a concentration of

Table 8. Seasonal changes in the simple lipids of Sia-Varaz Orange juice sacs. Graphical representation of the data is shown in Figs. 22-24. The values are expressed as mg/100 g powder

Lipid	Aug	Sept	Oct	Nov	Dec	Jan	Feb	Mar
MG	18.8 ef	18.4 e	19.2 efg	20.0 efgh	13.4 cd	11.1 c	6.8 a	7.2 ab
DG	27.1 gh	26.6 g	22.1 de	10.4 a	13.1 ab	16.7 c	20.4 d	22.1 def
FS	66.7 efg	64.0 bcde	60.6 bcd	46.0 a	52.8 ab	57.4 bc	66.6 def	89.5 h
Unk	8.3 fg	8.5 fgh	8.0 f	6.0 de	4.0 c	5.2 cd	2.6 a	2.7 ab
FFA	8.9 a	9.8 ab	12.5 abc	16.7 d	17.4 de	27.5 f	34.6 g	38.7 h
TG	85.9 cdef	86.0 cdefg	85.9 cde	88.6 cdefh	76.7 cd	73.1 c	58.5 a	60.4 ab
SE	99.7 fgh	99.5 fg	98.8 f	79.4 ab	79.4 abc	83.3 abcde	68.6 a	80.8 abcd
HC	14.9 ab	15.4 abc	12.5 a	27.1 d	47.3 e	54.8 f	62.7 g	69.4 gh
Total simple lipid	330.3 cdefg	328.2 cde	319.6 bc	294.2 a	304.1 ab	329.1 cdef	320.8 bcd	370.8 h
Simple lipid percentage to total lipid	19.1	19.4	19.9	19.4	20.5	22.8	23.4	25.8
Total lipid	1729	1689	1598	1519	1479	1443	1405	1436

See table 10 p 155 for complex lipids

330 mg/100 g powder in August (Table 8) making up 19.1% of total lipid (Fig. 21). There was a marked decrease in November followed by a significant increase in January and March, reaching a maximum of 371 mg/100 g powder in March.

In order to discuss these changes, changes occurring in the individual simple lipids during the development cycle of the fruit must be taken into account. Fig. 17 shows the simple lipids present in orange juice sacs were:

Monoglycerides (MG)

The monoglycerides showed no significant change from August (19 mg/100g powder) to November (Table 8 and Fig. 22) but then decreased substantially in December (13 mg/100 g powder) with no apparent change in January. There was another decrease in February (7 mg/100 g powder) with no significant change during March. It appears that there was a sudden change in the biochemical activities in the orange between November and December which coincides to an extent with the transition from the cell enlargement stage of development to the maturation stage.

Diglycerides (DG)

There was a fluctuation in the diglycerides level throughout development. The concentration of this lipid remained constant in the first two months of the

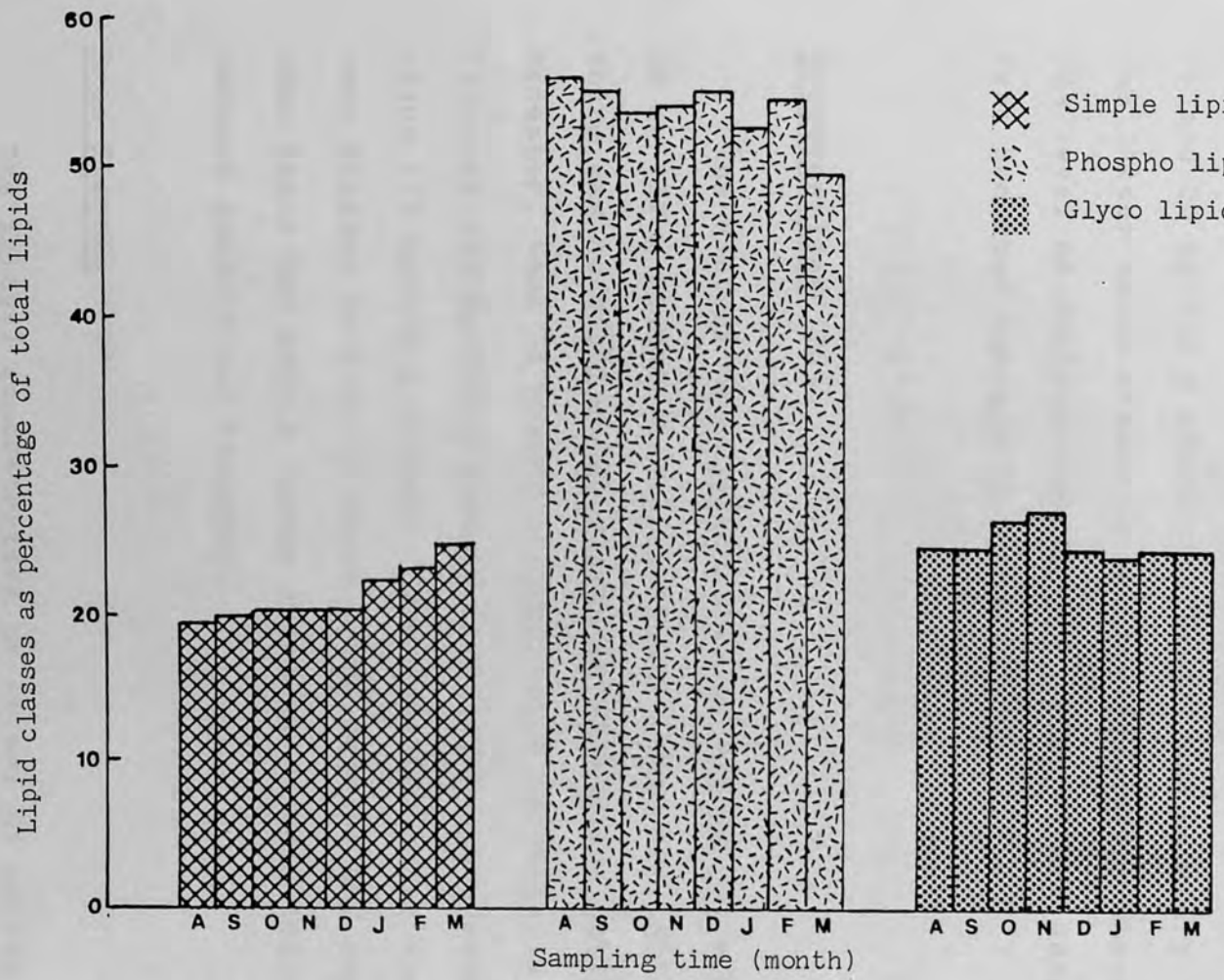


Fig. 21. Seasonal changes in the percentage weight of lipid classes of Sia-Varaz orange juice sacs

experiment (27 mg/100 g powder respectively) (Table 8 and Fig. 22), and then decreased substantially to 22 and 10 mg /100 g powder in October and November respectively. No marked change was observed in December (13 mg/100 g powder). The concentration of this lipid increased significantly to 17 and 20 mg/100 g powder in January and February respectively. The latter value showed no substantial change during March. The level of diglycerides showed a significant increase from December through to March.

Triglycerides (TG)

The content of triglycerides in orange juice sacs was 86 mg/100 g powder in August (Table 8 and Fig. 22), and there was no substantial change in this value until December, then it showed a significant decrease in February (59 mg/100 g powder) compared to the January value (73 mg/100 g powder). The February value was very similar to that of March. The results may suggest that there had been a sudden increase in cell metabolism between January and February.

Free Sterols (FS)

The initial concentration of FS was 67 mg/100 g powder (Table 8 and Fig. 23) with no obvious changes in September and in October but then a substantial decrease to 46 mg/100 g powder took place in November. The

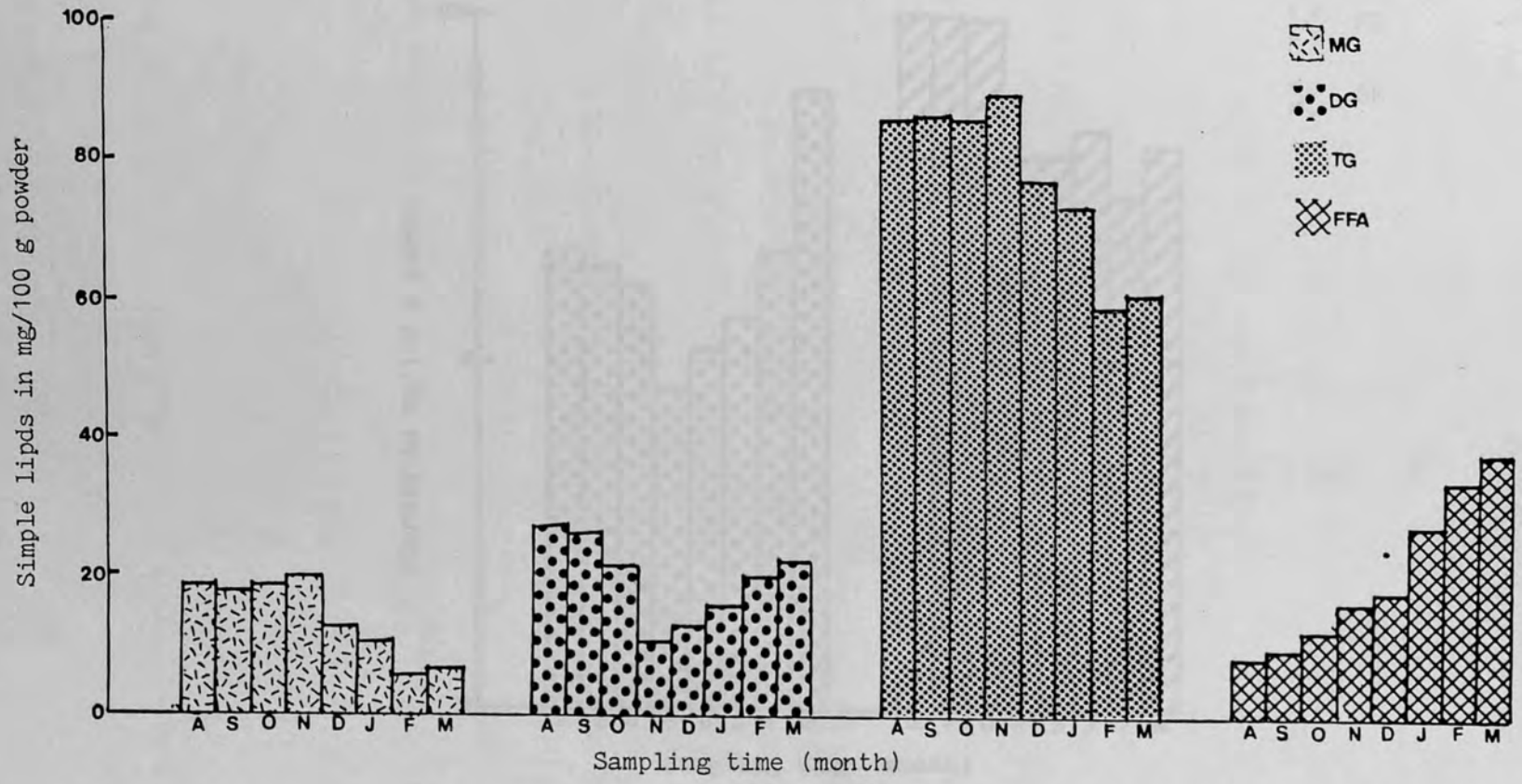


Fig. 22. Seasonal changes in the simple lipid contents of Sia-Varaz orange juice sacs. For significant differences refer to Table 8

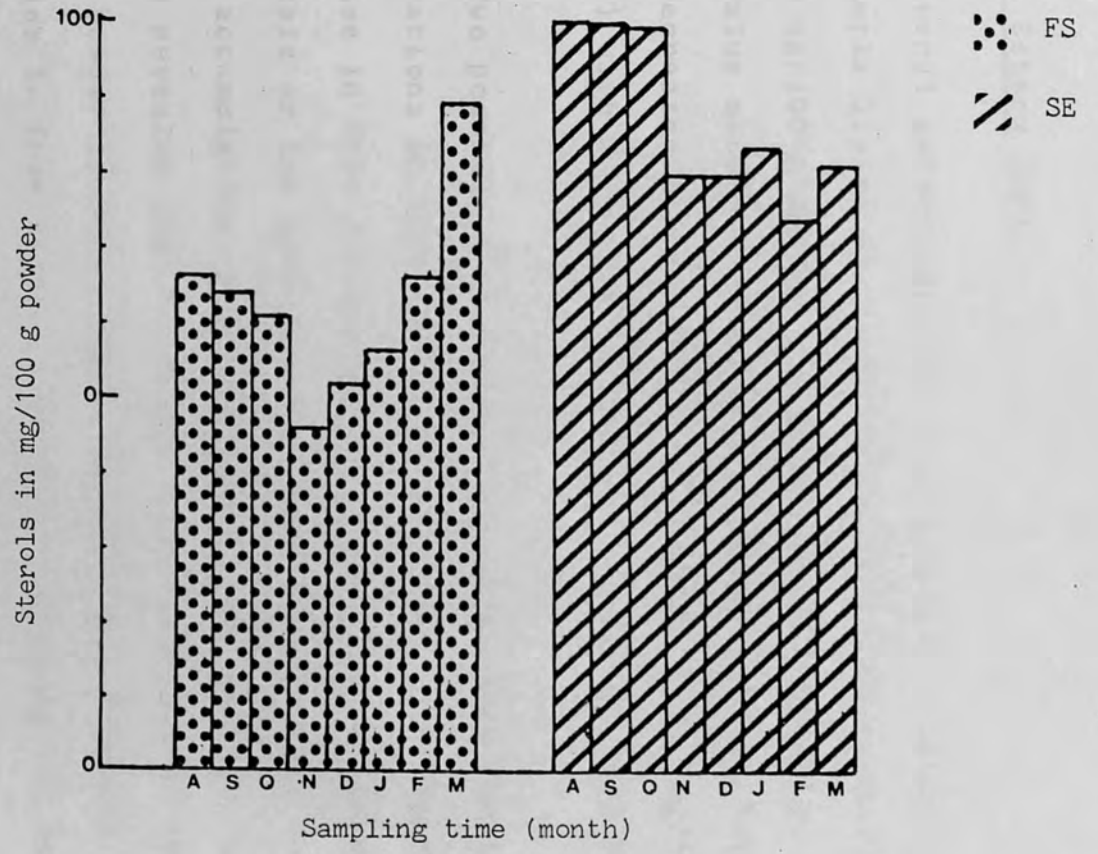


Fig. 23. Seasonal changes in the free sterols and steryl esters of Sia-Varaz orange juice sacs. For significant differences refer to Table 8

trend, albeit slight, was upwards with a significant increase in February and in March when they reached the maximum of 90 mg/100 g powder.

Steryl Esters (SE)

Steryl esters were the most abundant fraction within the simple lipids of orange juice sacs with a concentration of 100 mg/100 g powder in August (Table 8 and Fig. 23). This value showed no significant change until October, then decreased substantially in November (79 mg/100 g powder) with no marked changes in the months that followed.

Two possible explanations may be offered for the observations on free and esterified sterols. The net increase in free sterols may have been due to increased synthesis or the hydrolysis of steryl esters may have led to an accumulation of free sterols. The results for steryl esters revealed that there was a net decrease of 19 mg/100 g powder by March which could account for most of the increase in free sterols, thereby supporting the second explanation.

Hydrocarbons (HC)

The amount of hydrocarbons was relatively low in immature fruits (15 mg/100 g powder) in August (Table 8) and stayed at that level until November and December when it rose to 27 mg/100 g powder and to 47 mg/100 g powder

respectively. Between December and January there was another significant increase in their concentration, reaching 55 mg/100 g powder in January. This value increased to 63 mg/100 g powder in February with no substantial changes in March.

The significant increase in concentration of hydrocarbons between October and January supported the previous observation relating to colour break of the peel (Chapter 1). This occurred in November, was due to loss of chlorophyll leading to unmasking of underlying pigments, and the synthesis of new pigments.

Total free fatty acids have shown a large increase over the eight month period of development (Table 8 and Fig. 22). The initial concentration of total free fatty acids was 9 mg/100 g powder which showed no significant change between September and October. This was followed by a significant increase in November (17 mg/100 g powder) with no marked change in December (17 mg/100 g powder). The concentration of free fatty acids was increased substantially to 27, 35 and 39 mg/100 g powder in January, February and March respectively.

A part of this increase may be attributed to the hydrolysis of the mono-, di-, and triglycerides, as previously observed.

A clearer picture may emerge if free fatty acid content and composition are studied more closely. This will be discussed now.

Individual Free Fatty Acids

The fact that total simple lipid has shown a net increase was due partly to the increase in individual free fatty acids (Table 9, Fig. 24). If the results are considered as a percentage of individual fatty acid to total fatty acids (Table 9) it is found that the percentage of C14:0 was very low at the initial development stage and at maturation, but it was measurable during cell enlargement. In contrast to this, both C18:0 and C16:0 showed constant decreases. The percentage of C18:0 was measurable initially in the immature fruit but decreased to trace quantities during development. The percentage content of C18:1 and C18:2 during development displayed fluctuating trends, in that C18:1 initially decreased and then increased while C18:2 showed no set pattern. Linolenic acid (C18:3) was the only fatty acid showing a steady increase throughout development.

During the first stage (i.e. cell division) there was a gradual build up of C18:3 through a chain of reactions which probably led from C16:0 with desaturating reactions leading to polyunsaturated molecules. This was reflected in the observation that C16:0, C18:0 and C18:1 all showed an initial decrease while both C18:2 and C18:3

Table 9. Seasonal changes in the free fatty acids of Sia-Varaz Orange juice sacs. Graphical representation of the data is shown in Fig. 24.

Sampling time (month)	Total free fatty acids (mg/100 g powder)	Fatty acids in mg/100 g powder						Percentage of fatty acids					
		C14:0	C16:0	C18:0	C18:1	C18:2	C18:3	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3
Aug	8.9 a	T	2.7 a	0.1 n.s.	2.2 a	3.0 a	0.9 a	-	30.3	1.2	24.5	33.4	10.6
Sept	9.8 ab	T	2.9 b	0.1 n.s.	2.4 ab	3.3 ab	1.0 ab	-	30.0	1.1	24.2	34.0	10.7
Oct	12.5 abc	0.1	3.7 bc	0.1 n.s.	3.1 abc	4.2 abc	1.3 abc	0.5	29.6	0.7	25.1	33.3	10.8
Nov	16.7 d	0.1	4.8 d	T	4.1 d	5.9 d	1.8 cd	0.6	28.5	-	24.9	35.1	10.9
Dec	17.4 de	0.1	4.8 de	T	4.6 de	5.9 de	2.0 cde	0.3	27.9	-	26.5	33.7	11.6
Jan	27.5 f	T	7.6 f	T	8.0 f	8.2 f	3.6 f	-	27.8	-	29.2	29.7	13.3
Feb	34.6 g	T	9.4 g	T	10.2 g	10.0 g	4.9 g	-	27.2	-	29.6	29.0	14.2
Mar	38.7 h	T	10.4 h	T	11.6 h	10.4 gh	6.2 h	-	27.0	-	30.0	27.0	16.0

n.s. = not significant

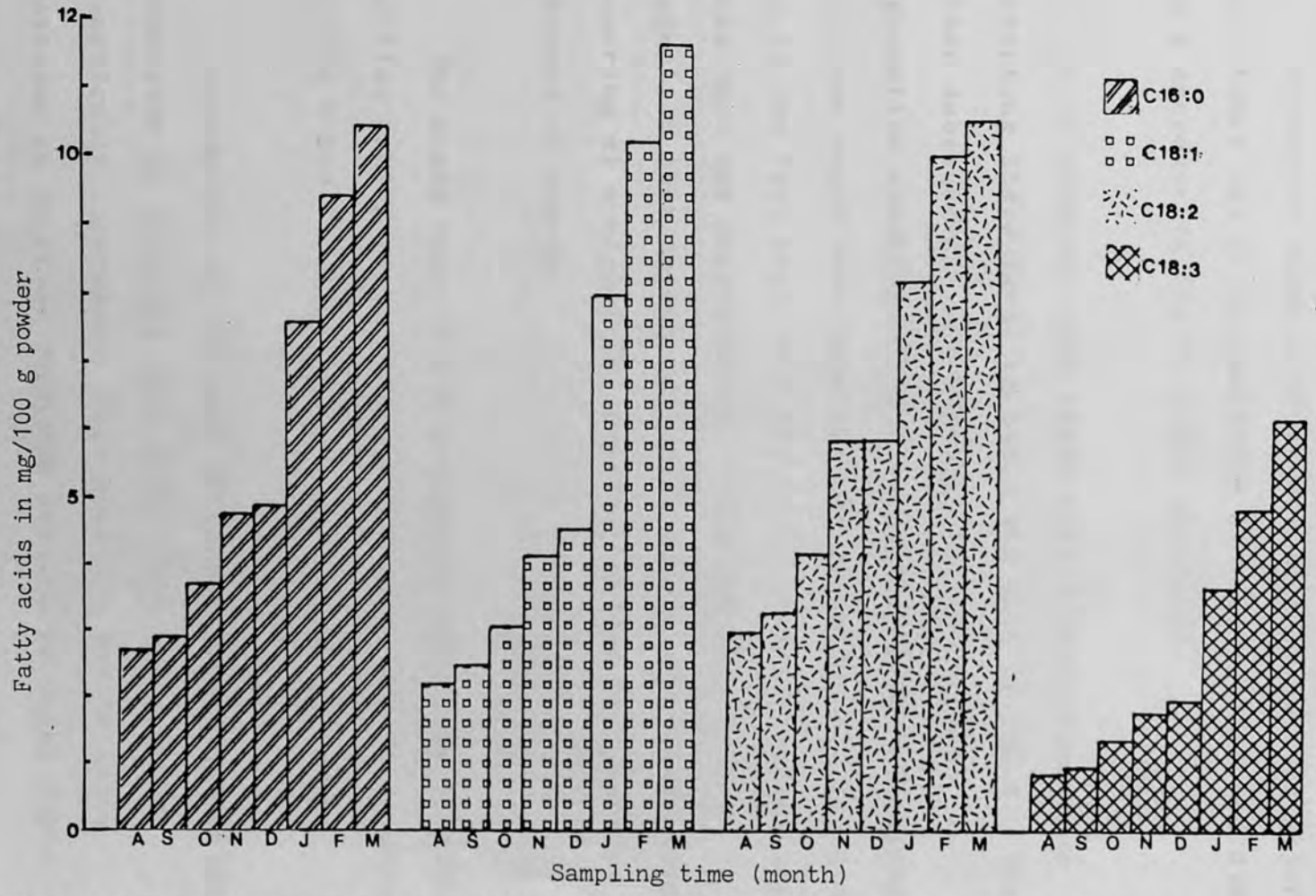


Fig. 24. Seasonal changes in the major free fatty acid contents of Sia-Varaz orange juice sacs. For significant differences refer to Table 9

showed an initial increase.

Throughout stage 2 of development (i.e. cell enlargement) there was an accumulation of C14:0, C18:1 and C18:3 and a decrease in C16:0, C18:0 and C18:2.

It is possible that there were enzymic reactions converting C18:2 first to C18:1 and then to C18:0. The latter subsequently was degraded to C16:0. The degradation reaction of 18:0 → 16:0 must be proceeding at a more rapid rate than the reduction of 18:2 to 18:0 due to the fact that 18:0 was found in trace quantities while 18:1 was accumulating. This may lead to the suggestion that during cell enlargement respiration was occurring at a high rate, possibly utilising lipids as a source of energy.

The steep rise in C18:3 suggests that some anabolism was also taking place, hence C18:2 must also be diverted to form C18:3.

Towards the end of cell enlargement and throughout maturation it appeared that total free fatty acids dramatically increased. This would be due to lipid breakdown as observed from the results of total lipid content (Table 8) and discussed earlier (p.140).

Complex Lipids

The complex lipid fraction in the orange juice sacs was the largest fraction of total lipids. It made up 80.9% of total lipids in August (Table 10). Of this, phospholipids made up 56.2% of total lipids and glycolipids only 24.7% (Table 10 and Fig. 21).

In general, total complex lipids in the orange juice sacs decreased significantly during development and maturation. Phospholipids appeared to be more affected than glycolipids in that net decrease in the former was 258 mg/100g powder compared to only 74 mg/100 g powder in the latter. Both values were shown to be significant. The decrease in total phospholipids was significant between October and November, after which there was a levelling off during December and January followed by another decrease in March.

However, on considering total complex lipids as a percentage of total lipids (Table 10) it was observed that this value stayed at approximately 80% until December. Throughout January and up to March this value decreased to approximately 74%, indicating that some complex lipids may have been broken down or converted to simple lipids.

Table 10. Seasonal changes in the complex lipids of Sia-Varaz Orange juice sacs. The values are given as mg/100 g powder. Graphical presentation of the data is shown in Figs. 25 and 26

	Aug	Sept	Oct	Nov	Dec	Jan	Feb	Mar
Phospho-lipids								
PC	352.0 defgh	342.0 def	343.0 defg	300.8 d	323.6 de	221.0 bc	206.0 ab	177.0 a
PE	302.5 gh	297.0 g	229.1 cdef	221.1 bcde	186.9 abcd	185.0 ab	186.0 abc	162.0 a
PA	121.3 gh	109.0 g	50.9 ab	61.0 abc	48.9 a	78.0 de	76.0 d	80.0 def
PS	51.3 a	52.0 ab	67.0 cdefgh	61.0 abc	62.3 abcdef	65.0 cdefg	60.0 abc	62.0 abcde
PI	47.9 a	48.9 ab	50.9 abc	54.9 abcdef	57.6 cdefg	57.6 cdefgh	54.0 abcde	52.0 abcd
PG	44.0 a	46.0 ab	61.0 c	70.4 cd	77.0 de	85.0 ef	101.0 g	107.0 gh
LPC	32.3 fgh	30.0 efg	25.5 cde	28.8 def	22.1 abc	24.8 bcd	19.0 a	21.0 ab
UNK	20.9 abc	18.8 a	30.1 d	20.1 ab	30.8 de	46.0 f	46.0 fg	52.0 h
Glyco-lipids								
MGDG	251.2 n.s.	255.0 n.s.	262.6 n.s.	261.3 n.s.	265.3 n.s.	247.0 n.s.	232.0 n.s.	242.0 n.s.
CMH	175.2 fgh	162.0 fg	158.8 ef	145.4 e	100.5 a	105.0 abc	104.0 ab	110.0 abcd

n.s. = not significant

/continued.....

Table 10 continued...

	Aug	Sept	Oct	Nov	Dec	Jan	Feb	Mar
Total phospho-lipid	972.2 gh	943.7 g	857.5 def	818.1 bcde	809.2 bcd	762.4 abc	748.0 ab	714.0 a
Total glyco-lipid	426.4 efgh	417.0 ef	421.4 efg	406.7 de	365.8 abcd	352.0 abc	336.0 a	352.0 ab
Total complex lipid	1398.6 gh	1360.7 fg	1278.9 def	1224.8 de	1175.0 bcd	1114.4 abc	1084.0 ab	1065.0 a
Phospho-lipid % to total lipid	56.2	55.3	53.6	53.8	54.7	52.8	53.2	49.7
Glyco-lipid % to total lipid	24.7	24.7	26.4	26.8	24.7	24.4	23.9	24.5
Complex lipid % of total lipid	80.9	80.6	80.0	80.6	79.4	77.2	79.2	74.2
Total lipid	1728.9	1688.9	1598.5	1519.0	1479.1	1443.5	1404.8	1435.8

Phospholipids

These will be discussed in order of decreasing relative amount.

Phosphatidyl Choline (PC)

This complex lipid was the most abundant phospholipid in the August sample (352 mg/100 g powder) Table 10 and Fig. 25). Its concentration did not change significantly for the first three months of sampling, then decreased substantially in November with no significant change in December, but followed by a significant decrease in January. There was another decrease in March. The total net loss of this lipid was 175 mg/100 g powder and was found to be a significant loss.

Phosphatidyl Ethanolamine (PE)

Phosphatidyl ethanolamine was the second most abundant phospholipid in August with a concentration of 303 mg/ 100 g powder (Table 10 and Fig. 25). This decreased significantly in October (229 mg/100 g powder). In general, the results indicated a gradual decrease during cell enlargement and maturation causing a significant loss of 140 mg/100g powder by March.

Phosphatidic Acid (PA)

The concentration of this lipid was high in the first two months of this study (Table 10 and Fig. 25), then

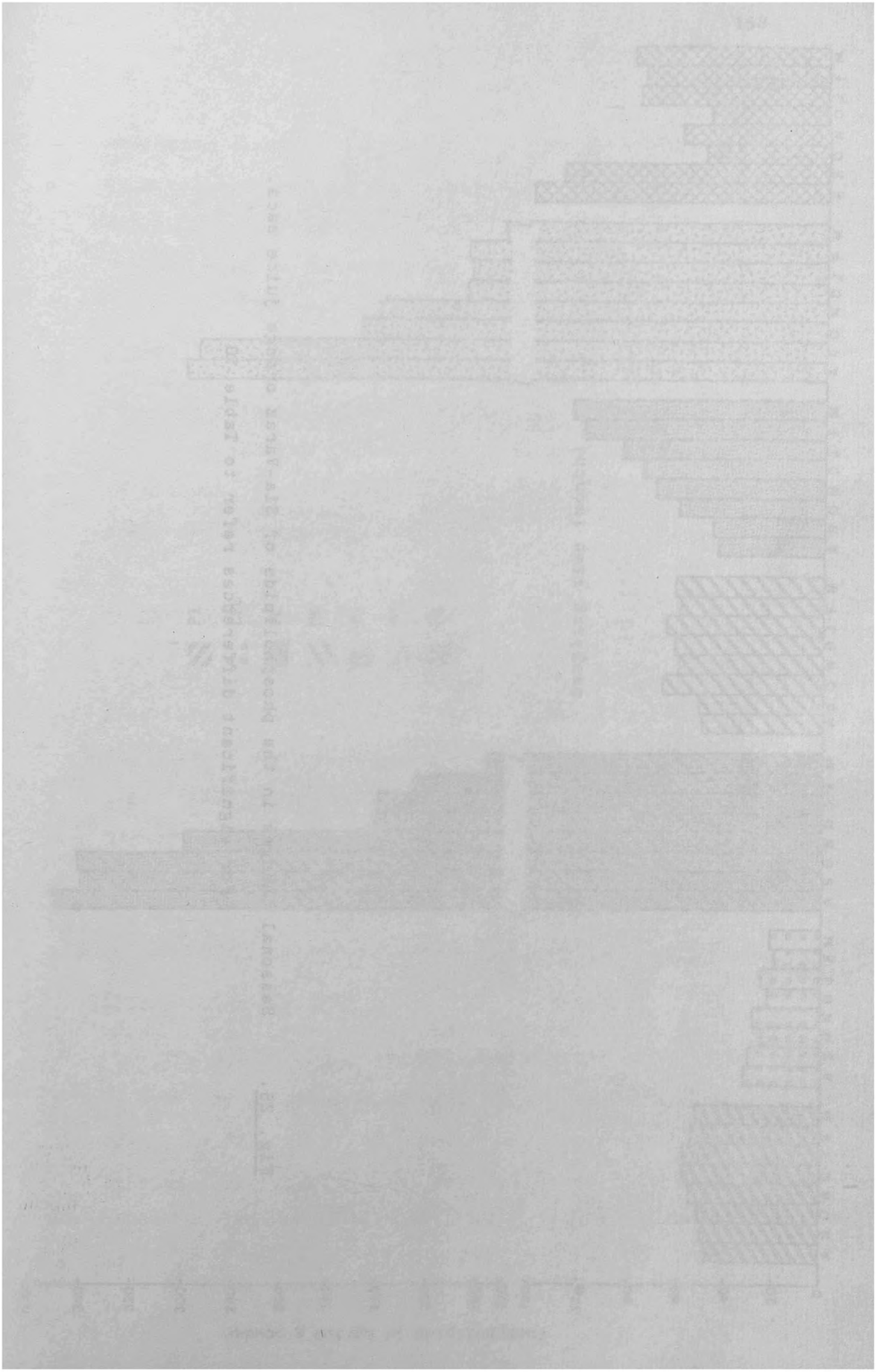
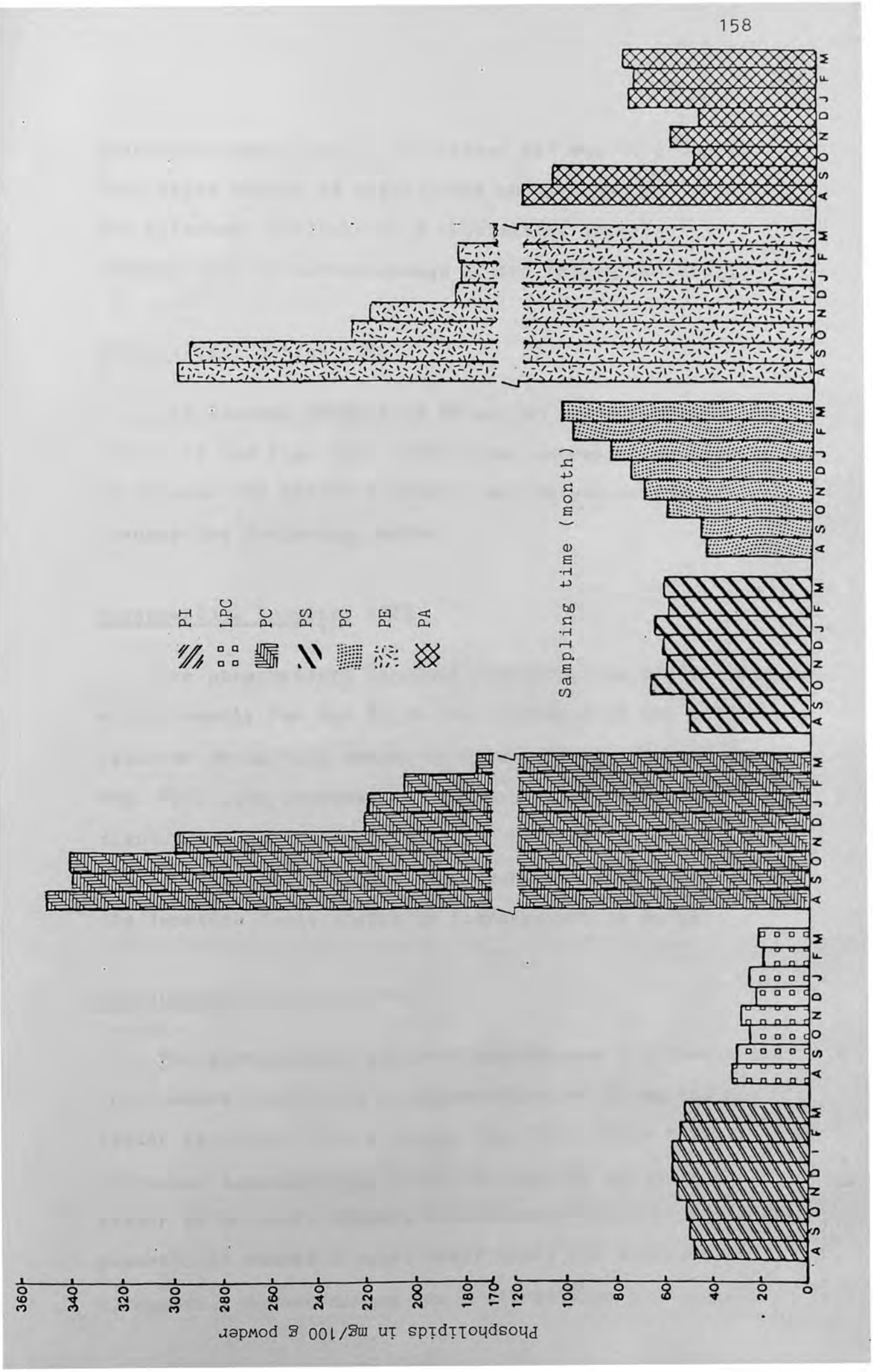


Fig. 25. Seasonal changes in the phospholipids of Sia-Varaz orange juice sacs.

For significant differences refer to Table 10


Phospholipids in mg/100 g powder
0
20
40
60
80
100
120
170
180
200
220
240
260
280
300
320
340
360
A S O N D J F M
Sampling time (month)
PI
LPC
PC
PS
PG
PE
PA

decreased significantly in October (51 mg/100 g powder). This value showed no significant changes between October and December, followed by a substantial increase in January with no marked change in the following months.

Phosphatidyl Serine (PS)

The initial content of PS was 51 mg/100 g powder (Table 10 and Fig. 25). This value increased substantially in October (67 mg/100 g powder) and showed no significant changes the following months.

Phosphatidyl Inositol (PI)

The phosphatidyl inositol concentration did not change significantly for the first four months from its initial value of 48 mg/100g powder in August (Table 10 and Fig. 25). The December and January values showed a significant increase compared to those of the first two months of study, followed by a decrease to the level of the immature fruit stages in February and in March.

Phosphatidyl Glycerol (PG)

The phosphatidyl glycerol content was relatively low in immature fruit with a concentration of 44 mg/100 g powder in August (Table 10 and Fig. 25). This value increased substantially to 61, 85 and 101 mg/100 g powder in October, January and February respectively. In general, it showed a significant total net increase of 63 mg/100 g powder during the fruit development cycle.

Lysophosphatidyl Choline (LPC)

This derivative of PC showed a concentration of 32 mg/100 g powder in August (Table 10 and Fig. 25). Its content did not change significantly between August and November but decreased substantially in December (22 mg/100 g powder) with no marked changes in the following months. As an overall concentration it showed a substantial decrease of 9 mg/100 g powder by March.

Unknown Phospholipids

The substantial increase in the total net amount of the unknown phospholipid throughout the sampling and its tentative identification as a phospholipid containing at least one amino group, suggested that it may be a derivative of PE, possibly the lyso form of the PE.

Glycolipids

Glycolipids are the predominant amphipathic lipids of plant membranes and the major lipid constituents in chloroplasts. Two major glycolipids identified in Sia-Varaz orange juice sacs were Monogalactosyl diacylglycerol (MGDG), and Ceramide monohexosides (CMH).

Monogalactosyl Diacylglycerol (MGDG)

This glycolipid was the third most abundant lipid of the complex lipid fraction with a concentration of

251 mg/100 g powder in August (Table 10 and Fig. 26). However, the results showed that there was a slightly downward trend (not significant) in its concentration during the development cycle of the fruit, reaching 242 mg/100 g powder by March.

Ceramide Monohexoside (CMH)

The concentration of CMH was 175 mg/100g powder in August (Table 10 and Fig. 26). It showed no substantial change between August and November. This was followed by a significant decrease in December with no significant changes the following months.

Total Fatty Acids

The fatty acids C14:0 and C18:0, which were in small but measurable amounts during the analysis of free fatty acids, were found to be in larger amounts in total fatty acids (Table 11). This suggested that these two fatty acids were mostly esterified to different groups of lipids. The predominant fatty acids of total lipids were found to be C16:0, C18:1, C18:2 and C18:3. The relative percentage of the latter were very similar to that found among the free fatty acids (Table 9).

Myristic acid (C14:0) and stearic acid (C18:0) both showed a fluctuating pattern during development and maturation of the fruit.

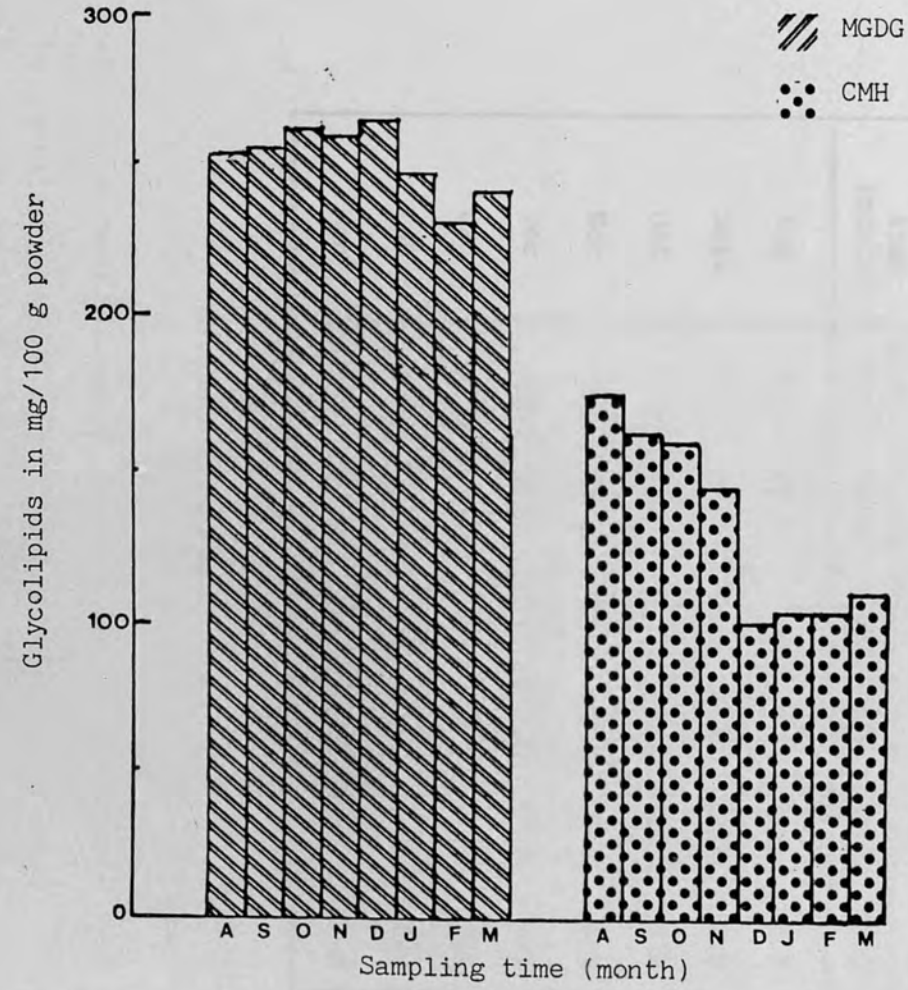


Fig. 26. Seasonal changes in the glycolipid contents of Sia-Varaz orange juice sacs. For significant differences refer to Table 10

Table 11. Seasonal changes in the relative percentage of fatty acids of total lipids of Persian Orange juice sacs

Sampling time (month)	Percentage of fatty acids					
	14:0	16:0	18:0	18:1	18:2	18:3
Aug	6.0	30.0	1.0	23.7	34.2	5.1
Sept	5.6	29.3	0.9	24.0	33.7	6.5
Oct	2.9	31.4	1.4	25.6	31.3	7.4
Nov	5.4	30.0	0.8	26.7	30.2	6.9
Dec	4.0	30.8	0.2	27.3	31.3	6.4
Jan	4.3	27.5	0.3	27.6	32.9	7.4
Feb	4.8	26.7	1.0	26.6	33.0	7.9
Mar	4.2	25.2	0.4	25.6	37.3	7.3

Palmitic acid (C16:0) showed a continuous increase in the first three months of sampling, then decreased significantly the following months.

Oleic acid (C18:1) tended to increase gradually throughout cell division and cell enlargement, but decreased during maturation, whereas C18:2 behaved in direct contrast. This suggested that during the first two stages of fruit development there was reduction of C18:2 to C18:1, while the opposite occurred during maturation. Linolenic acid (C18:3) showed a significant increase in the first three months of sampling, then decreased substantially during cell enlargement and finally increased in the maturation period.

DISCUSSION

Fig. 16 (p. 111) shows that PA was the parent compound of the glycerophosphatides. In the present study the loss in complex lipids have largely occurred in PC, PE, and in CMH. The largest decrease was in PC, some of which could have been converted to PS leading to the observed increase in the latter. Between December and January PA showed a significant rise, hence it was equally possible that some PC may have been interconverted to PA. The significant decrease in PA content between September and October coincided with a substantial increase in PG, /

indicating that the latter may have arisen from PA conversion through CDP-Diglyceride.

From the results of simple and complex lipids it appears that there was a substantial change in the biochemical activities of the orange fruit pulp somewhere between November and December. Baine and Mercer (1967) have reported that during the maturation period of almost all fruits, there is a disorganisation of chloroplast when the chloroplast lamella breaks down and the constituents of the membrane, both lipids and proteins, are broken down by enzymic reaction (Rhodes and Wooltorton, 1967). After breakdown of the lipoprotein membrane, fatty acids are released from many unbound complex lipids by lipolytic acyl hydrolases. In addition, phospholipase D will attack phospholipids, causing hydrolysis of this group of lipids (Gaillard, 1975).

Sinclair (1961) and Davis (1970) have found that respiration of orange fruits increased during maturation.

From the results in Tables 8 and 10, it was observed that mono-, di-, and tri-glycerides decreased to some extent while phospholipids (the main constituents of membranes) have decreased the most. The presence of substantial amounts of free fatty acids as a consequence of lipid breakdown strongly suggests that these free fatty acids are available for cell respiration causing the onset of maturity in the fruit.

Although there were changes in different lipid constituents, there was, however, no definite pattern or trends to distinguish between the stages of cell division, cell enlargement and maturation. However, partial evidence has been offered in support of the onset of maturation, from changes in phospholipids and in simple lipids reported between November and March.

CHAPTER VI

GENERAL CONSLUSION ON DEVELOPMENT AND MATURATION OF THE SIA-VARAZ ORANGE

General Characteristics

The development cycle of the orange was found to exist in three separate stages. These were termed Cell Division, Cell Enlargement and Maturation respectively. Cell Division occurred between August and September, Cell Enlargement spanned from October to December while Maturation lasted from January to March.

The greatest increase in size of the orange was observed during Cell Division and Enlargement (118%) compared to the subsequent increase during Maturation (5.6%).

Colour break from yellow to orange occurred between December and January. The environmental temperature during these months ranged from 12.1^{o} - 7.0^{o}C respectively.

The largest volume of juice accumulation occurred between October and December, during cell enlargement.

A continual drop in total acidity was noted through-

out development. The maximum decrease occurred between October and December which was probably due to the dilution effect from the increase in juice volume. Acidity is known to be affected by temperature, hence the observed low value may have been due to the high temperatures the oranges were subjected to during their early stages of development (22°C between June and August).

Total soluble solids showed a continuous increase throughout development. This was mainly due to the synthesis of free sugars which made up approximately 80% of total soluble solids in the fully mature orange juice.

The ratio of total soluble solids to acid is often used as an index to measure maturity of the orange or its suitability for picking from the tree. The Iranian Regulations require the ratio to be 10:1, while the Florida Citrus Growers recommend that the percentage weight of total free sugar be seven times the weight of total acid for the fruit to be termed mature. Taking the above into account, the Sia-Varaz orange was deemed mature for picking as early as January if necessary.

However, another factor to be taken into account would be consumer preferences. Bayton and Bell (1954) have studied consumer preferences and have found that among those who generally liked juices, there was a high

preference for solid/acid ratios ranging from 12-18. Persian oranges most reflecting this value were picked in February and in March.

The total soluble solids to acid ratio was not very reliable as the only factor for determining maturity. Other factors such as free sugars, free amino acids and lipid content and their relative changes were also considered.

Free Sugars

Five free sugars were identified, namely sucrose, glucose, fructose, galactose and xylose. Xylose was only found in early samples (August and September), while galactose existed in minor quantities. Sucrose and reducing sugars increased with fruit development but glucose and fructose never reached the same level as that of sucrose. The maximum increase in free sugar content occurred during cell enlargement. Sucrose remained the most abundant single sugar throughout the season. In fact, the ratio of sucrose:glucose:fructose was very close to 2:1:1 as was also shown by Curl and Veldhuis (1948) and by Tingard Attaway (1971).

Total free sugar content of the mature Sia-Varaz orange juice used in this study was found to be superior to that of other varieties such as Valencia (Harding et al., 1940; Ting, 1954), Hamlin and Pineapple (Ting, 1954).

This quality or property makes the Sia-Varaz orange a useful contributor for blending with other juices, in order to enhance their sugar content.

Vitamin C

The vitamin C content measured as mg/100 g pulp showed no significant change throughout development, from 54 mg/100 g pulp in August to 50 mg/100 g pulp in March. Although these values were slightly higher than those reported by Harding et al. (1940) working on Valencia oranges, they followed the same general trend.

The results expressed as mg vitamin C/orange pulp showed that the vitamin C content increased from 15 mg/pulp in August to 57 mg/pulp in March. A sharp increase was observed between October and December which coincided with the large increase in the weight of the fruit.

Two suggestions were offered in order to explain the fairly high vitamin C content in the Sia-Varaz variety of orange used: (i) that the climate in the Caspian Region between October and March provided optimum conditions for ensuring high vitamin C content, and (ii) the Sia-Varaz oranges used in this study were picked from the exposed part of the orchard. This latter resulted in increased photosynthetic activity in the plant thereby increasing the sugar content, leading to increased vitamin C manufacture.

The daily consumption of one mature Sia-Varaz orange (i.e. picked in March) provides 57 mg of vitamin C intake to the body. This fulfils the minimum conditions set by the FDA (1973) for RDA, thereby providing enough vitamin C for good health in an adult.

Free Amino Acids

Seventeen free amino acids were identified. These were present in varying amounts, and any differences found in the Sia-Varaz orange appeared to be quantitative rather than qualitative.

During the complete cycle of development (August to March), a steady but significant increase was noted from an initial content of 178 mg/100 ml to 529 mg/100 ml. The rate of increase was substantially increased during Cell Enlargement and slowed down during Maturation.

The predominant amino acids in the earlier samples were aspartic, threonine, serine, glutamic acid and proline. Arginine and alanine were also found in appreciable amounts, these becoming predominant in the mature orange. The ammonia content of the juice appeared to decrease during development, probably being used for the synthesis of amino acids.

Glutamic acid, the most abundant amino acid in the immature fruits, was the only one showing a net decrease

in mature oranges. Proline and arginine showed the largest net increase of 111 and 114 mg/100 ml respectively. An interesting observation was that both of these amino acids demonstrated sharp increases between December and January, coinciding with the onset of maturation. This was also noted by Clement and Leland (1962b).

It was suggested that these huge increases in proline and in arginine between December and January may be used as an indication for determining the initiation of the Mauration Stage in Sia-Varaz oranges.

Lipids

From the results of individual and total lipids, it seems that there was a substantial change in biochemical activities of the orange fruit pulp between November and December. Among the complex lipids, phosphatidyl choline, phosphatidyl ethanolamine, phosphatidic acid, and cerebrosides decreased the most. It was suggested that PC may have been interconverted to phosphatidic acid and to phosphatidyl serine.

Although there are changes in different lipid constituents, in general there is no definite pattern or trend to distinguish between the stages of cell division, cell enlargement and maturation. However, partial evidence was offered in support of the onset of maturation,

from the biochemical activities and changes reported between November and December.

Because of the rapidity with which biophysical and chemical changes take place in fruit tissue during maturation and ripening, it is essential that there should be close contact between grower and processor in order to determine the optimum stage of ripeness for use in a specified type of process and to determine the time at which the fruits should be harvested.

The processor mainly relies upon empirical tests of organoleptic qualities, and decisions on harvesting time are usually based upon experience, reinforced by the results of batch processing.

Specified changes in composition have been proposed as the basis of assessment of the onset of ripening in the Sia-Varaz orange. However, timing for the harvesting of fruit is further complicated by the differences which occur in the rate of development and maturation of individual fruits even on the same tree. This problem of uneven ripening becomes accentuated when mechanical harvesting is used, where the personal discrimination exercised in hand picking is absent. The use of hormones in promoting an even maturation is likely to become of increasing value, provided other quality characteristics are not adversely affected.

The variety of oranges used in this study appears to have great potential in juice processing with regard to its high vitamin C and sugar contents, coupled with low acidity of the juice.

SECTION II

CANNING AND STORAGE OF SINGLE-STRENGTH ORANGE JUICE

CHAPTER VII

GENERAL INTRODUCTION

Fruits have traditionally played an important part in man's diet. In the early part of this century it was strictly a seasonal item which could only be consumed in the fresh state. The earliest science of food preservation began to develop with the discovery that foods would not support microbial growth and decomposition if most of the water was removed. With the advent of modern processing methods an even larger variety of flavourful and health products became available to the consumer throughout the year. This is especially noticeable in fruits and fruit-related products. This has resulted in a steady increase in the consumption of processed fruits and fruit juices.

Fruit juices are consumed for the flavour and nutritional properties. Fruit juices are a good source of vitamin C, providing about 35% of this vitamin to the diet. In addition, they are an aid in regulating the body's elimination processes.

Food preservation provides a flavoured, nutritious and attractive product that will retain these qualities during extended storage. A better standard of living is experienced today because of the year-round availability

of high quality nutritious foods. Processing can destroy some food nutrients, as can also cold and warm temperature storage. The amount of change depends upon many factors, such as maturity, variety, conditions of the fruit, as well as the processing conditions, container type, storage temperature and duration of storage.

In this study the focus was placed on the effect of canning and canned storage at different temperatures on single-strength orange juice. Naturally, in evaluating new or modified procedures leading to improved nutrient retention, product acceptability must be kept in mind. The advantages of better nutritional quality are nullified if the product is less acceptable to the consumer. The factors of acceptability include those of colour, flavour and texture towards which the nutritional constituents of the canned juice play a large contribution. In order to assess the storability of canned orange juice, information would need to be obtained on the loss and chemical interactions of those nutrients under long-term storage conditions. In particular, no work to date has been reported on the effect of canning and canned storage on the Sia-Varaz variety of sweet orange.

The object of this study was therefore to process single-strength Sia-Varaz orange juice expressed from the same batch of mature fruits as discussed in Section I. The juice was then stored at two temperatures (7^{o} and $30^{o}C$)

for one year. The effect of processing and storage of the canned juice at 7° and at 30°C on various nutrients will be the subject of Section II.

GENERAL MATERIALS AND METHODS

Canning of Orange Juice

The problem with the fresh juice is not only that of micro-organisms but also of naturally occurring enzymes in the foodstuff which could, during storage, have deleterious effect on palatability. Because citrus juices have lower pH values than the majority of other foods, milder conditions of processing are effective in their preservation. Heating to temperatures of only 65.5°C will destroy most spoilage organisms but some heat resistant molds (Byssochlamys fulva and B. nivea) may require pasteurization temperatures as high as 98.9°C for control (Nagy and Attaway, 1980).

Desirable physical characteristics of well processed citrus juices are that they retain the cloud of fresh juice and remain free of gel lumps. The cloud, composed of heterogenous particles of pectin, protein and lipid, is derived from the rag and pulp of squeezed oranges suspended in the clear aqueous serum (Baker and Bruemmer, 1972). The cloud may form gel lumps on storage by enzymic de-esterification and degradation of suspended pectin by the action of pectinesterase. This enzyme, however, may be destroyed by pasteurization.

Orange juice contains a sufficient amount of pectinesterase which may demethylate pectin during or after

processing unless heat is applied to inactivate this enzyme. Atkins et al. (1956) and Kew et al.(1957) observed that heating citrus juices to about 70°C prevented fermentation, but 86-99°C was required to stabilize the cloud. Following these studies, the current commercial practice was developed. This consists of rapidly heating the juice to about 90-100°C, holding at that temperature for a fraction of a minute, then rapidly cooling the canned product. To achieve this, the juice is kept hot as it comes from the pasteurizer, is pumped to a filler bowl and filled directly into cans (the filling temperature should be maintained at a minimum of 88°C and the cans should be completely filled with the hot juice). When adequate filling temperatures are maintained, atmospheric closure is satisfactory for canned juice. The cans are closed automatically and inverted for about one minute to sterilize the lid by the heat of the juice, and then are rapidly cooled by cold water to 35-40°C. No processing is necessary when the juice is filled to provide a minimum closing temperature of 88°C, providing the cans are 12 oz. or larger. If the cans are smaller than 12 oz., they should be processed for 10 minutes at 100°C (Lopez, 1981).

Procedure

In the present study the fully matured oranges were used for extracting the juice for canning. Extra care was taken to avoid corrodible metal contact during extraction of the juice. The fruits were cut into halves with a

stainless-steel knife. Half of each fruit was lightly hand-reamed. Coarse pulp, seeds, and much of other suspended matters were removed by screening through a 30 mesh stainless-steel sieve. Thereafter the juice was only in contact with glass containers which had been chemically cleaned and rinsed thoroughly with distilled water.

Pasteurization was carried out in a glass container by heating the juice to 98°C and holding it at this temperature for 30 seconds.

A1 (300 ml) enamel-lined (lacquered) cans were filled completely with hot juice and enamel-lined lids were placed on top of the cans. The cans were closed by seaming the lids. After closing, the cans were inverted for one minute and rapidly cooled in cold water. The "exhausting" step was not needed since the cans were completely filled with hot juice, hence steam displaced any residual air. Eight cans were taken for determining the direct effect of processing on vitamin C, free sugar, free amino acids, and lipids.

Two batches of 40 cans each were taken. One of these batches was stored in the cold room at 7 $\pm$ 1°C while the other batch was stored in the incubator at 30 $\pm$ 1°C. These were used for further analysis in order to evaluate the effect of storage over a 12 months period on free sugars, optical density, formation of 5-hydroxymethylfurfural

(Chapter VIII); on vitamin C, formation of furfural, organoleptic tests (Chapter IX); free amino acids (Chapter X); and on lipids (Chapter XI).

CHAPTER VIII

EFFECT OF CANNING AND CANNED STORAGE ON FREE SUGARS, OPTICAL DENSITY, AND HYDROXYMETHYLFURFURAL

INTRODUCTION

The sugars, which contribute much to the acceptability of citrus juices, may under adverse conditions be altered to produce off-flavours that reduce the acceptability of the citrus juices or their products. The sugars, primarily monosaccharides, can participate in browning reactions causing darkening of the juice and giving rise to components that are described generally as apricot-like or pineapple-like in flavour (Nagy and Attaway, 1980).

Curl (1949) in a study conducted with synthetic orange juice, reported that darkening of the synthetic juice occurred principally when both amino acids and sugars were present. Wolfrom et al. (1974), using model systems involving sugars and amino acids, showed that glucose and fructose had considerable effects on browning in the presence of different amino acids. Their results also showed that sucrose had no direct effect on darkening of the juice. Pruthi and Lal (1951) in a study of different

methods for preserving and storing citrus juices, reported that addition of 5% cane sugar to the juices accelerated the darkening of the juice.

Berry et al. (1971) found that the stability of foam-mat dried instant orange juice, stored at 21° and 29°C, could be improved by the use of more acidic juices or by removing sugars. Increasing the acidity probably aids in slowing down the Maillard reaction, subsequently slowing down colour development.

There are two general types of complex reactions occurring during the storage life of fresh and preserved foods where sugars and amino acids are present. These are the dehydrating reactions favoured by acidity (Wolfrom et al., 1948, 1949) and the Maillard type of non-enzymic browning reactions (Reynolds, 1965) taking place between the amino acids (free or combined as proteins or peptides) and the reducing sugars. Both reactions are considered to arise through a 3-deoxyhexulose type of intermediate (Fig. 27).

In summary, in the Maillard type reaction (Fig. 28) a variety of groups such as aldehydes, ketones and reducing sugars, combine with amino groups in aldol condensation to form first a Schiff's base and then a N-substituted glycosylamine. These compounds undergo Amadori rearrangement, at which stage the compounds are still colourless and the

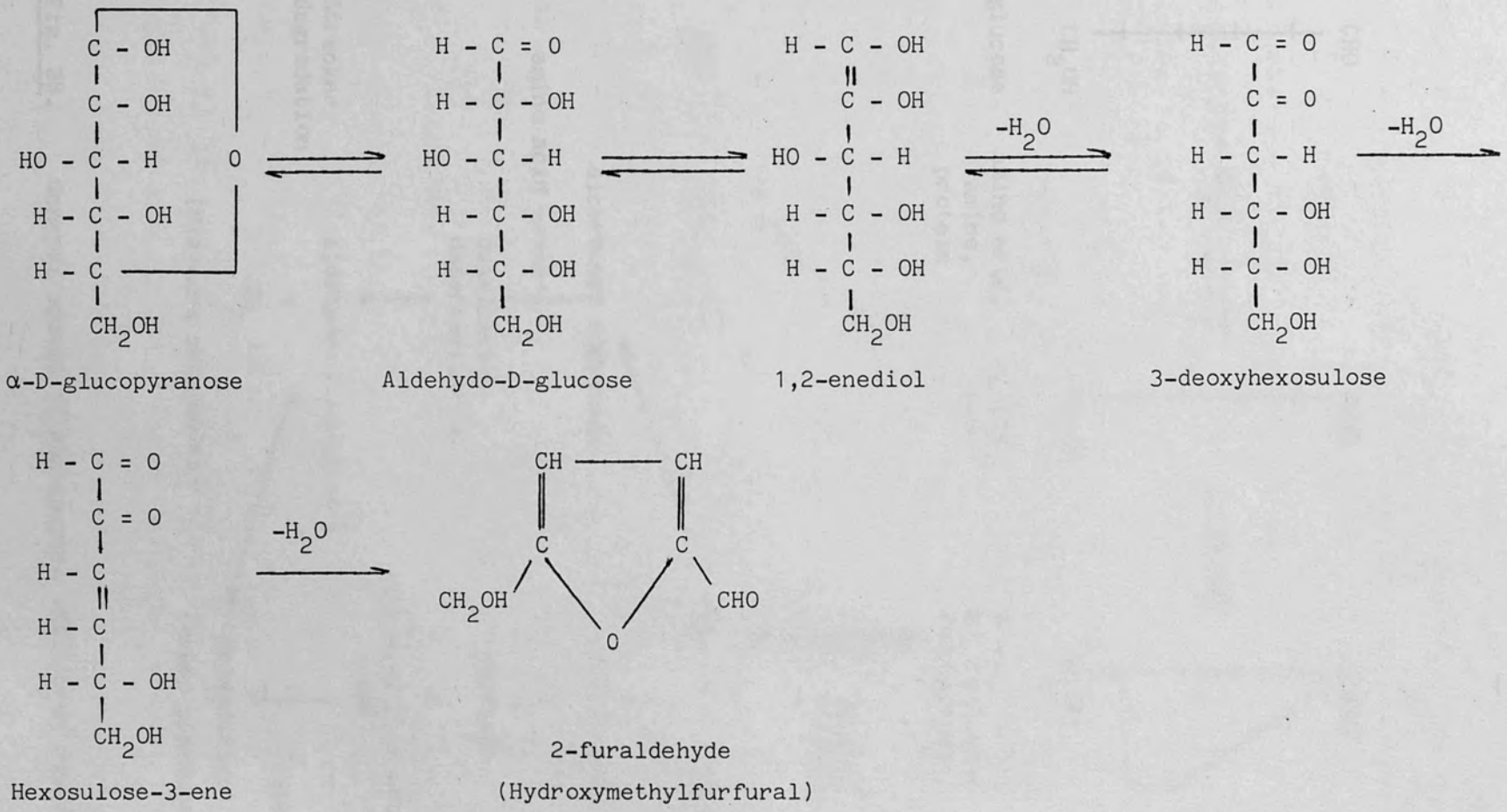


Fig. 27. Dehydration reactions of hexose under acidic conditions proposed by Shallenberg and Birch (1975)

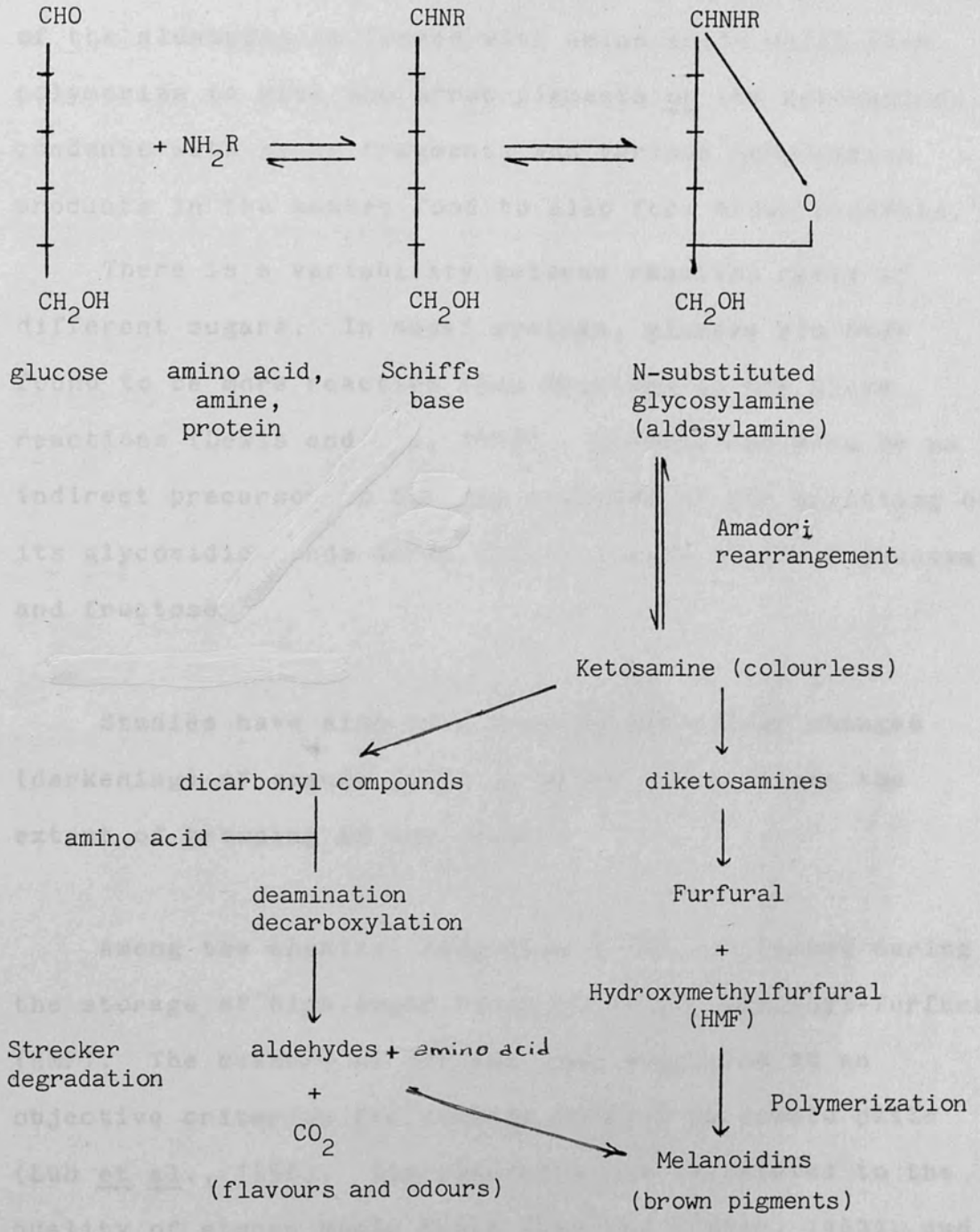


Fig. 28. General scheme of non-enzymic (Maillard) reactions

The third stage is either a Strecker degradation with loss of a molecule of carbon dioxide, followed by condensation of the aldehydes so formed with amino acids which then polymerize to give the brown pigments or the ketosamines condense with sugar fragments and various dehydration products in the heated food to also form brown pigments.

There is a variability between reaction rates of different sugars. In model systems, glucose has been found to be more reactive than fructose in the above reactions (Lewis and Lea, 1959). Sucrose can also be an indirect precursor to this as a result of the splitting of its glycosidic bonds in an acid solution to yield glucose and fructose.

Studies have also been made on the colour changes (darkening) of orange juice in order to elucidate the extent of browning of the juice.

Among the chemical compounds which are formed during the storage of high-sugar foods is 5-hydroxymethyl-furfural (HMF). The measure of HMF has been suggested as an objective criterion for storage changes in tomato paste (Luh et al., 1958). Its concentration is related to the quality of stored apple sauce (Luh and Kamber, 1963), and storage at lower temperature results in a slower rate of HMF formation. Both processing temperature and storage temperature affect the HMF content of honey (White et al., 1964). These findings suggested that HMF may be formed

during the canning and canned storage of orange juice.

Tatum et al. (1967) have mentioned that many compounds such as HMF are formed by acid degradation of sugars. Berry and Tatum (1965) have studied the effect of storage on foam-mat dried orange juice powder and found the formation of HMF in stored powder. They concluded that HMF is not responsible for the flavour considered characteristic of stored orange powders, its detection may be the first indication of storage changes. More recently, Tatum et al. (1975) have found HMF as one of the degradation products formed in canned single-strength orange juice after storage at 32°C for twelve weeks. Therefore, quantitative studies have also been made to measure HMF content to reflect storage changes.

MATERIALS AND METHODS

Determination of Optical Density of Orange Juice

The formation of brown complexes and their development was measured directly from the increase in absorption at a set wavelength according to the method devised by Meydav et al. (1977). In this method 50 ml of single-strength orange juice was centrifuged at 2,000 r.p.m. for 20 minutes to remove pulp and coarse cloud particles. The supernatant was diluted 1:1 (V/V) with 95% ethanol and filtered through Whatman No. 42 filter paper, to obtain a fully clarified extract.

The absorbance of the extract was measured at 420nm on a spectrophotometer. Five replicates of each clarification procedure were used for absorbance measurements.

Quantitative Study of 5-Hydroxymethylfurfural

To extract hydroxymethylfurfural the method described by Tatum et al. (1975) for canned single-strength orange juice was used. In this method, 500 ml of canned single-strength orange juice was transferred to a 1,000 ml beaker, and 50 g sodium chloride was added and the solution mixed for 5 minutes using a magnetic stirrer. The juice was then divided between two 500 ml separatory funnels. Each portion was extracted five times with 100 ml methylene chloride for

each extraction. The extracts were dried over anhydrous sodium sulphate, combined and evaporated to dryness on a rotary evaporator at 30°C. The residue was dissolved in 1 ml of ether and acetone mixture (1:1, V/V). This solution was examined by TLC.

The TLC procedure described by Berry and Tatum (1965) was used for separating degradation products of orange juice. In this procedure, 10 µl of prepared solution was placed on a plastic backed Silica gel G plate (0.25 mm thick) alongside pure 5-hydroxymethylfurfural and the plate was developed in a mixture of benzene, ethanol, water and acetic acid (200:47:15:1, V/V) as described by Hay et al. (1963). After developing, the plate was air dried and examined under ultraviolet light for fluorescent spots. Hydroxymethylfurfural spots appeared as dark purple spots on the thin-layer chromatographic plate. These were marked, and then the plate was sprayed with anisaldehyde spray reagent in sulphuric acid as described by Bobbit (1963). The sprayed plate was heated to 160°C for 3.5 minutes for colour development. The HMF band appeared as a dark green colour (Fig. 29). One can then compare the colour visually by the intensity produced.

Anisaldehyde Reagent:

Half a milliliter of anisaldehyde was mixed with 0.5 ml concentrated sulphuric acid, 9 ml of 95% ethanol and a few drops of glacial acetic acid were added.

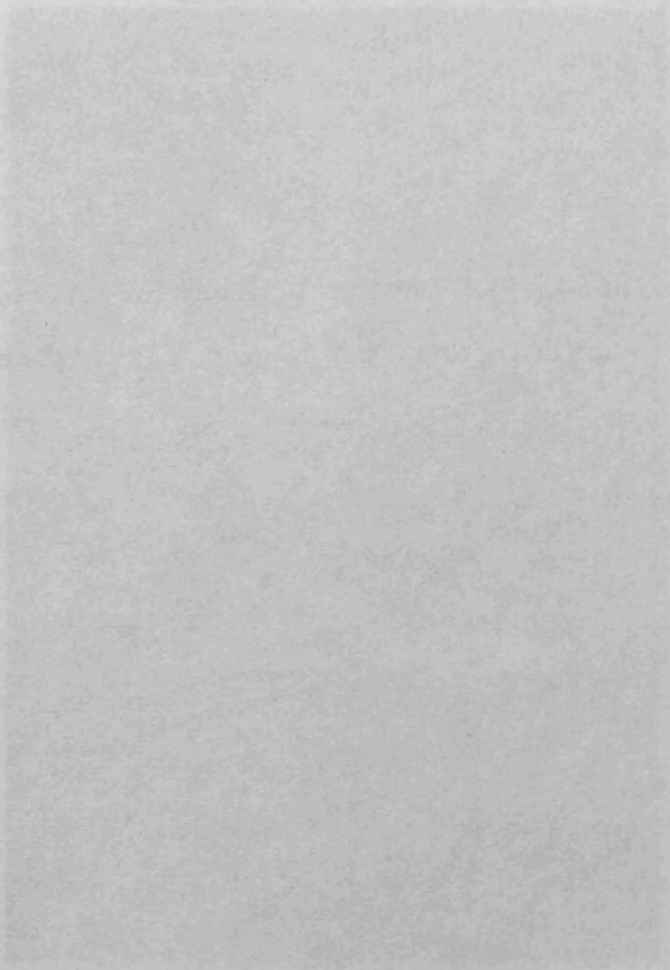
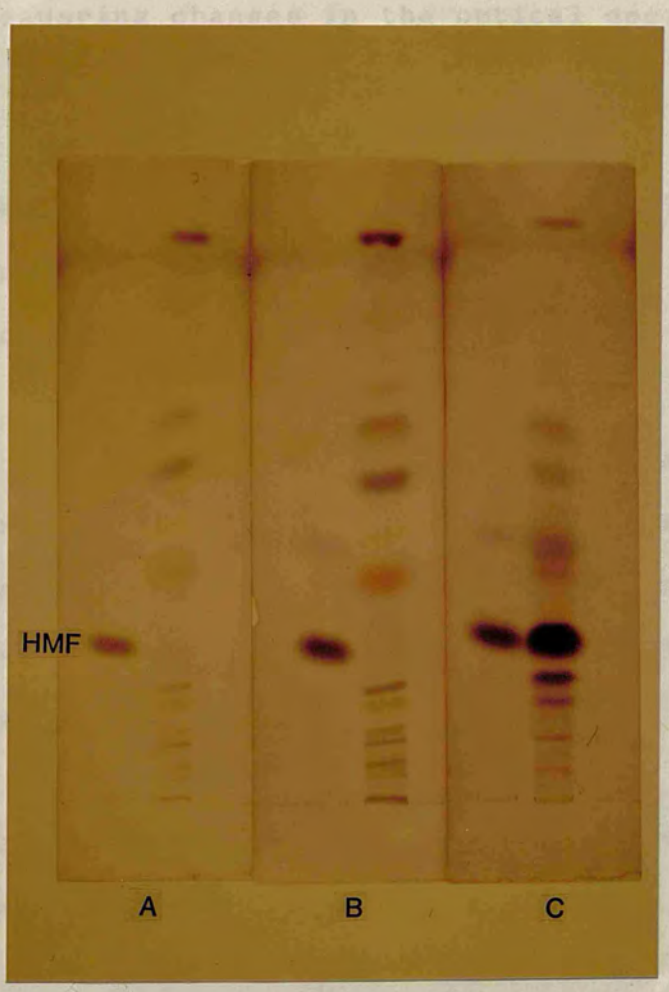


Fig. 29. Thin-layer chromatography of hydroxymethylfurfural of canned stored orange juice



A : fresh
B : stored at 7°C
C :stored at 30°C

RESULTS AND DISCUSSION

Darkening of Orange Juice

The colour increase was determined in processed orange juice by meausring changes in the optical density of the juice.

The results (Fig. 30) indicated that at 7°C storage temperature there was no change in the colour of canned orange juice stored for 12 months.

Passy and Mannheim (1979) studying changes in the opitcal density of canned grapefruit juice stored at various temperatures, also found that 5°C storage temperature of the juice resulted in no variation after nine months of storage.

Storage of canned orange juice for 12 months at 30°C resulted in a substantial increase in optical density. There was a lag period of one month followed by an increase to an almost constant rate. There was more than a five-fold increase in optical density. This implied that orange juice stored at 30°C for 12 months resulted in severe browning reactions (Figs. 30 and 31). The source of this colour was not determined.

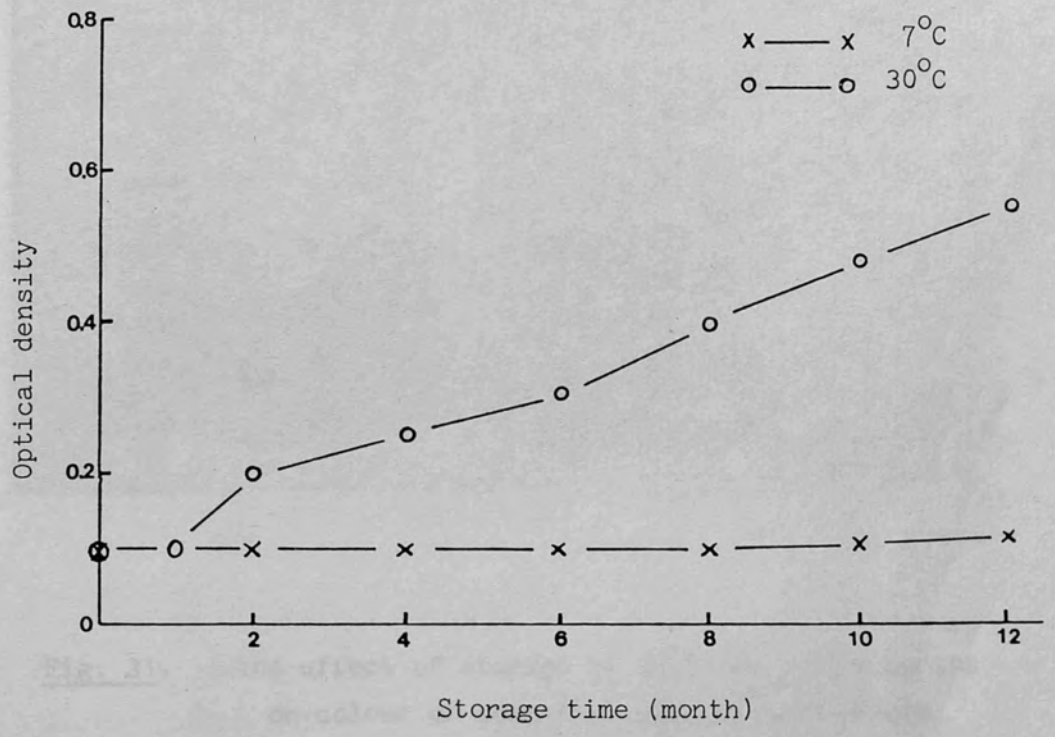


Fig. 30. The effect of storage over a twelve months period at two temperatures on the optical density of canned single-strength orange juice

Fig. 31. The effect of storage at 30°C for twelve months on colour of canned single-strength orange juice (B) compared to control (A)

Methods used for the determination of sugars were as those already described on p.60.

Effect of Canning

Total sugars in fresh orange juice and canned juice were recorded as 10.7 g/100 ml and 10.6 g/100 ml respectively, indicating no significant difference. The values for the individual sugar constituents were also constant (Table 12).

Effect of Canned Storage

Storage at 7°C of canned orange juice for 12 months has not led to any significant decrease in total sugar content; 10.6 to 10.0 g/100 ml (Table 13). Changes in individual sugar components have, however, taken place. Although galactose content has remained constant, sucrose content has dropped significantly from 4.9 to 2.9 g/100 ml indicating a loss of 2.0 g/100 ml juice. This corresponds to approximately 60% retention (Fig. 33). This loss has been continual throughout storage. Correspondingly, glucose and fructose have both shown continual increases during the 12 months of storage. Glucose content changed from 2.7 to 3.3 g/100 ml, representing an increase of 22%, while that of fructose changed from 2.8 to 3.6 g/100 ml, pointing to an increase of 29%. Both of these were signficant.

It appears that sucrose hydrolysis has been initiated under the acidic conditions prevalent in orange juice. This has further led to an increase in the component

Table 12. Effect of canning on free sugar contents of fresh Sia-Varaz orange juice

Orange juice	Free sugars (g/100 ml)				Total (g/100 ml)
	Glu	Fru	Suc	Gal	
Fresh	2.6	2.8	5.1	0.2	10.7
Canned	2.7	2.8	4.9	0.2	10.6

Table 13. Effect of storage at 7°C over a twelve months period on the free sugars of canned single-strength orange juice. Graphical presentation of the data is shown in Fig. 33

Sampling time (months)	Free Sugars									
	Glu		Fru		Gal		Suc		Total	
	g/100 ml	Retention %	g/100 ml	Retention %	g/100 ml	Retention %	g/100 ml	Retention %	g/100 ml	Retention %
0	2.7 a	100.0	2.8 a	100.0	0.2 n.s.	100.0	4.9 efg	100.0	10.6 n.s.	100.0
2	2.8 ab	103.7	2.8 ab	100.0	0.2 n.s.	100.0	4.7 ef	95.9	10.5 n.s.	99.0
4	3.0 abc	111.1	3.0 abc	107.1	0.2 n.s.	100.0	4.3 de	87.7	10.5 n.s.	99.0
6	3.2 abcde	118.5	3.2 abcd	114.3	0.2 n.s.	100.0	3.9 cd	77.5	10.4 n.s.	98.0
8	3.1 abcd	114.8	3.2 abcd	114.3	0.2 n.s.	100.0	3.6 c	73.5	10.1 n.s.	95.3
10	3.3 bcdef	122.2	3.4 abcde	121.4	0.2 n.s.	100.0	3.1 ab	63.3	10.0 n.s.	94.3
12	3.3 bcdef	122.2	3.6 cdef	128.6	0.2 n.s.	100.0	2.9 a	59.2	10.0 n.s.	94.3

n.s. = not significant

monosaccharides, glucose and fructose. The total increase in the monosaccharides was calculated to be 1.4 g/100 ml juice indicating that 0.6 g/100 ml of sucrose loss remains unaccounted for.

As was mentioned earlier, free sugars are susceptible to browning reactions, hence this could be one source of loss for the sugars. There was, however, no colour change noted in the canned juice (7°C) determined by optical measurements. This is not altogether surprising due to the fact that a number of products of the browning reactions are known to be colourless, hence any increase in these would not necessarily result in an overall change in colour of the canned orange juice. The fact that a relatively small increase was noted in HMF content (Table 14) also reflects limited sugar dehydration to form this precursor of the Maillard reaction products.

The GLC of free sugars of canned stored orange juice is given in Fig. 32. Storage of canned juice at 30°C led to substantial changes in total sugar content (Table 15 and Fig. 33). There was a change from 10.7 to 7.7 g/100 ml after 12 months of storage, indicating a significant loss. This corresponded to a retention of 72%. The maximum loss was incurred within the first few months, being 1.4 g/100 ml. This was subsequently reduced to a value of 0.8 g/100 ml loss for each of the next four months period (i.e. 4-8 months and 8-12 months of storage).

Table 14. Effect of storage at two temperatures over a twelve months period on the hydroxymethylfurfural content of canned single-strength orange juice

Storage time (months)	Storage temperature	
	7°C	30°C
0	-	-
2	-	+
4	-	+
6	-	++
8	Trace	+++
10	Trace	+++
12	+	++++

- = none

+ to ++++ = presence with increasing intensity

Fig. 32. Gas-liquid chromatogram of free sugar methyl esters of canned stored orange juice at 30°C for twelve months

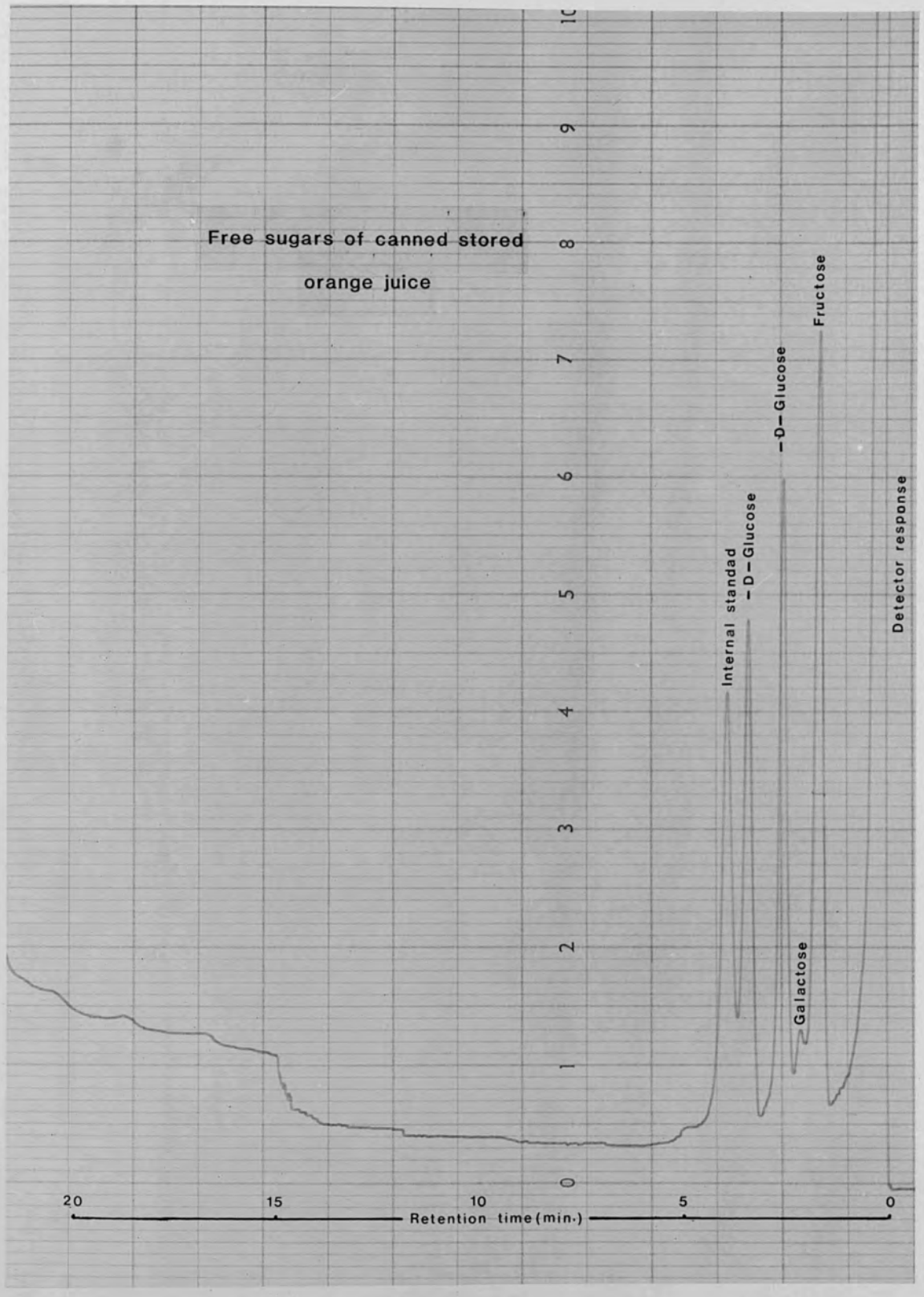

Free sugars of canned stored
orange juice
Internal standad
D-Glucose
D-Glucose
Fructose
Galactose
Detector response
0
1
2
3
4
5
6
7
8
9
10
20
15
10
5
0
Retention time (min.)

Table 15. Effect of storage at 30°C over a twelve months period on the free sugars of canned single-strength orange juice. Graphical presentation of the data is shown in Fig. 33

Sampling time (months)	Free Sugars									
	Glu		Fru		Gal		Suc		Total	
	g/100 ml	Retention %	g/100 ml	Retention %	g/100 ml	Retention %	g/100 ml	Retention %	g/100 ml	Retention %
0	2.8 a	100.0	2.9 a	100.0	0.2 b	100.0	4.9 e	100.0	10.7 fg	100.0
2	3.1 abcd	111.1	3.7 b	127.5	0.1 a	50.0	3.1 d	63.3	10.0 def	93.4
4	3.0 abc	107.1	3.9 bc	134.5	0.1 a	50.0	2.3 c	46.9	9.3 cde	86.9
6	3.3 cde	117.8	4.4 cd	151.7	0.1 a	50.0	1.1 b	22.4	8.9 abcd	83.2
8	3.4 cdef	121.4	4.6 de	158.6	0.1 a	50.0	0.4 a	8.2	8.5 abc	79.4
10	3.0 abc	107.1	4.9 defg	169.0	0.1 a	50.0	-	-	8.0 ab	74.8
12	2.8 ab	100.0	4.8 def	165.5	0.1 a	50.0	-	-	7.7 a	72.0

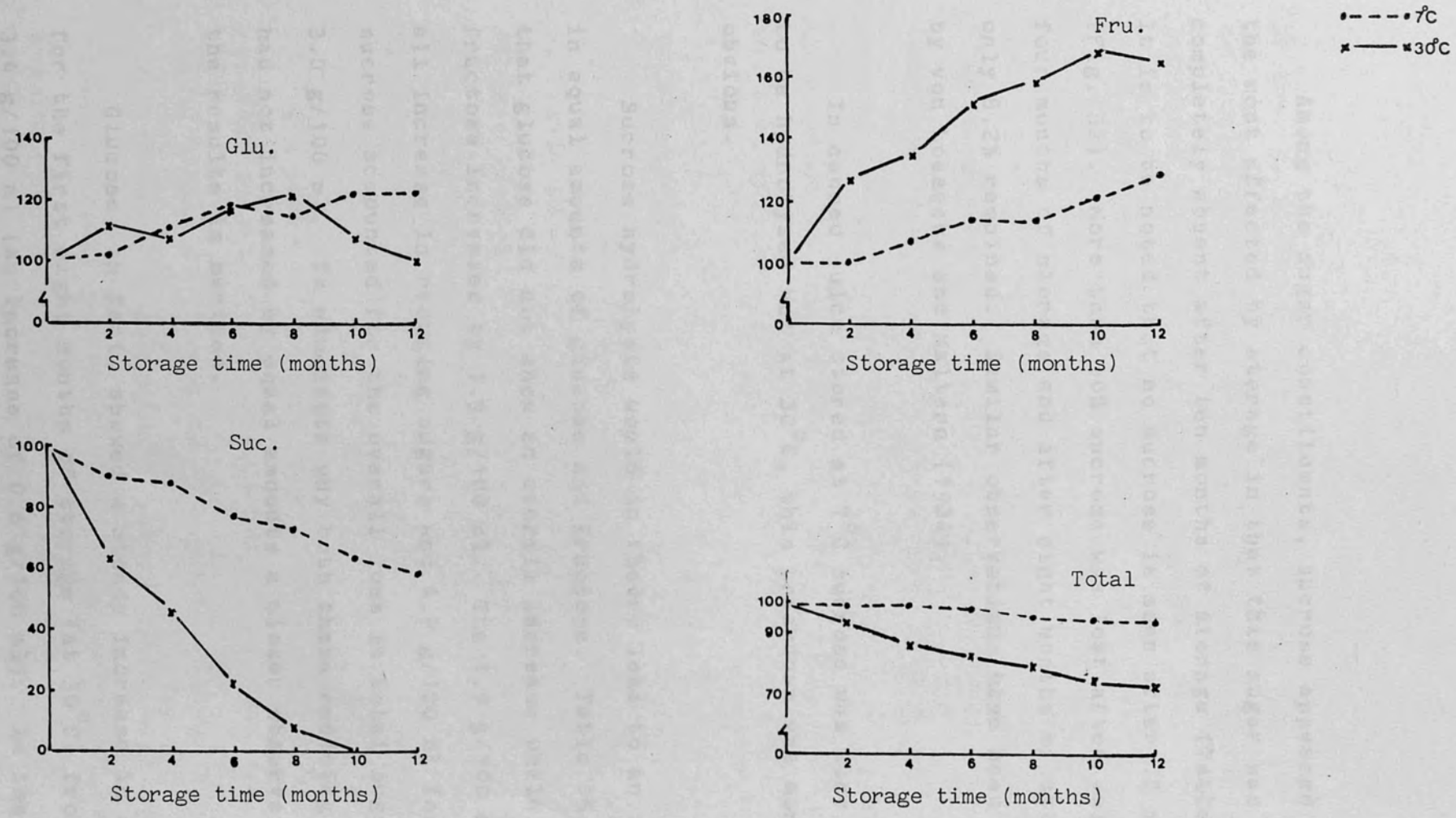


Fig. 33. Percentage retention of free and total free sugars of canned Sia-Varaz orange juice stored at 7° and 30°C over a twelve months period. For significant differences refer to Tables 13 and 15

Among the sugar constituents, sucrose appeared to be the most affected by storage in that this sugar was completely absent after ten months of storage (Table 15). It is to be noted that no sucrose is seen after 12 months (Fig. 32). More than 50% sucrose was lost after only four months of storage and after eight months of storage only 8.2% remained. Similar observations have been noted by von Loesecke and Maltern (1934).

In canned juice stored at 7°C sucrose was also observed to be hydrolysed but at 30°C, this breakdown was more obvious.

Sucrose hydrolysis would in theory lead to an increase in equal amounts of glucose and fructose. Table 15 shows that glucose did not show an overall increase while fructose increased by 1.9 g/100 ml. The 1.9 g/100 ml overall increase in reducing sugars and 4.9 g/100 ml loss in sucrose accounted for the overall loss in total sugars of 3.0 g/100 ml. To elucidate why both these reducing sugars had not increased by equal amounts a closer observation of the results is merited.

Glucose, in fact, showed a steady increase in content for the first eight months of storage (at 30°C) from 2.8 to 3.4 g/100 ml (an increase of 0.6 g/100 ml). In the same time interval of storage, fructose also showed an increase from 2.9 to 4.6 g/100 ml (an increase of 1.7 g/100 ml).

In theory, assuming that the total sucrose hydrolysed went to produce glucose and fructose, these sugars should have increased by approximately 2.2 g/100 ml. From the values shown above glucose is in deficit of 1.6 g/100 ml while fructose is 0.5 g/100 ml. This implies that these reducing sugars may have entered into further reactions. From the eighth to the twelfth month of storage glucose decreased from 3.4 to 2.8 g/100 ml, indicating a significant loss. Fructose, on the other hand, continued to increase to a value of 4.9 g/100 ml after ten months and then stayed constant.

After ten months of storage no more sucrose remained, hence the reducing sugar content would have been expected to remain constant. The fact that the latter decreased further supports the implication that the reducing sugars were being involved in further reactions. In these reactions glucose appeared to be more active than fructose.

The darkening of the juice at 30^{o}C storage was recorded as substantial, hence it is suggested that this colour change is related to the observed losses in the reducing sugars. Galactose found in small amounts showed a slight loss in the first two months and then remained constant throughout storage.

A combination of dehydration reactions leading to the formation of hydroxymethylfurfural, and direct involvement

of the reducing sugars in the Maillard reactions to form stable pigmented compounds appear to be the prime cause of loss of glucose and fructose. An accumulation of these pigments would also account for the observed darkening of the juice. This suggestion is derived from the fact that at 7°C storage the small loss of reducing sugars was not accompanied by darkening of the juice whereas the large losses recorded at 30°C storage resulted in darkening.

The fact that fructose loss is less than that of glucose also implies the occurrence of the Maillard reaction due to the fact that glucose is more susceptible to taking part in browning reactions (Adrian, 1982).

Sugars, however, would not be the only compounds causing browning (discussed in Chapter VIII) but it appears that they play quite an important part in this effect.

Another observation supporting the participation of sugars in causing browning of the juice is the noted increase in HMF content (Table 14), this being one of the precursors of the brown pigments formed in the Maillard reaction.

CHAPTER IX

EFFECT OF CANNING AND CANNED STORAGE ON VITAMIN C

INTRODUCTION

A major nutritional value of citrus fruit is their vitamin C content. Many nutritionists consider a daily intake of 46-80 mg per day as needed for maintaining good health. Vitamin C may be either consumed in the fresh form or after processing. Technology has presented various methods of processing the juice for consumption and these may have varying effects on the vitamin C content of the juice.

Since citrus fruits are considered one of the best sources of ascorbic acid, a considerable amount of work has been done on the retention of this vitamin during canning. Retention of vitamin C during the canning of orange juice has been reported by Moore et al. (1944); Lamb (1946), and Krehel and Cowgill (1950). All of these investigators have reported a uniformly high retention during canning. In general, the ascorbic acid content of orange juice appears to be little affected by canning. The retention of vitamin C potency in citrus products is important both to the consumer

concerned in maintaining good health, and to the citrus processor. In the 1940s, it was demonstrated that vitamin C in fresh and processed food was present mainly in one of its active forms, L-ascorbic acid (Mill et al., 1949; Guild et al., 1948). This is a relatively unstable molecule and is easily oxidised to dehydro-L-ascorbic acid which also has 100% antiscorbutic potency (Sebrell and Harris, 1967). The latter terminology refers to the active prevention of Scurvy, the disease caused by lack of vitamin C or its derivatives. Dehydro-L-ascorbic acid is also unstable and may be spontaneously converted to 2-3-diketo-L-gulonic acid which has no antiscorbutic potency (Mills et al., 1949); hence taking vitamin C derivatives in this form would not prevent Scurvy.

Ordinarily, orange juice in storage at room temperature deteriorates in flavour much more rapidly than other juices such as apple, pineapple, grape and tomato (Curl, 1949). This deterioration is generally accompanied by other changes, in particular darkening in the aqueous phase and losses of vitamin C. These may be caused by aerobic or anaerobic conditions.

The reaction pathways proposed by Bauernfeind and Pinkert (1970) for the degradation of vitamin C (Fig. 34) show that compounds such as furfural may be formed through anaerobic conditions while hydroxyfurfural is a product of the oxidative system.

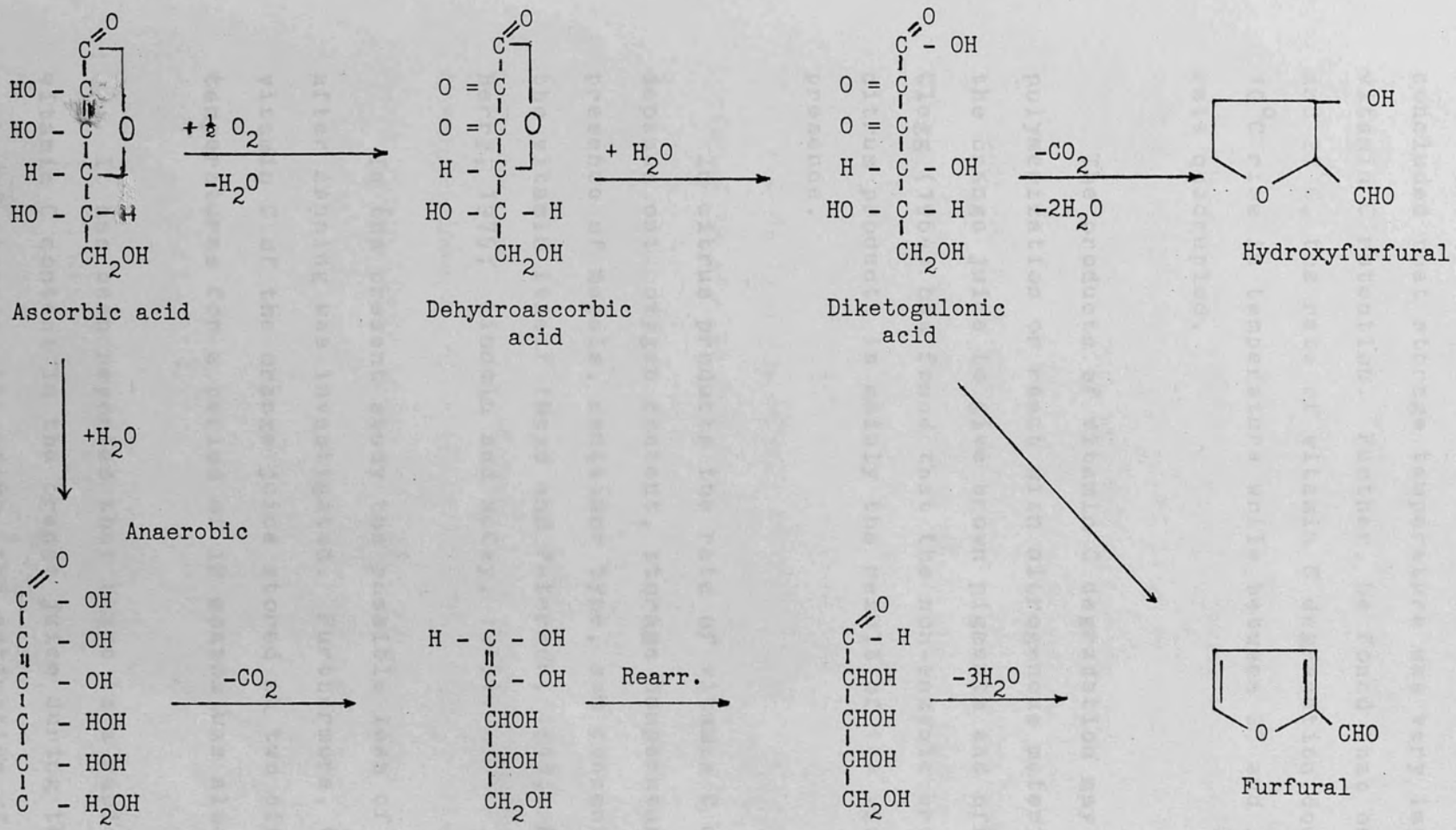


Fig. 34. Proposed ascorbic acid degradation pathways in the presence or absence of oxygen (Bauernfeind and Pinkert, 1970)

Ross (1944) has studied the flavour deterioration and vitamin C retention in canned orange juice and has concluded that storage temperature was very important to vitamin C retention. Further, he found that between 10 and 27°C, the rate of vitamin C degradation doubled for a 10°C rise in temperature while between 27 and 37°C the rate quadrupled.

The products of vitamin C degradation may undergo polymerization or react with nitrogenous materials within the orange juice to give brown pigments and off-flavour. Clegg (1964) has found that the non-enzymic browning in citrus products is mainly the result of the vitamin C presence.

In citrus products the rate of vitamin C degradation depends on: oxygen content, storage temperature, pH, presence of metals, container type, and concentration of the vitamin itself (Boyd and Peterson, 1945; Bissett and Berry, 1975; Lincoln and McCay, 1945).

In the present study the possible loss of vitamin C after canning was investigated. Furthermore, the loss in vitamin C of the orange juice stored at two different temperatures for a period of 12 months was also investigated.

It has been reported that there is a rapid decrease in vitamin C content in the orange juice during the first two weeks of storage, therefore, the estimation of vitamin C

was carried out weekly for the first two weeks, and then monthly for the rest of the storage period.

Furfural measurements were also taken as a further index of vitamin C breakdown. Huelin (1953) is of the opinion that the compound furfural originates from the decomposition of vitamin C. He conducted an experiment with 0.25% vitamin C in distilled water at 30°C for two years and found that the two major decomposition products were furfural and CO_2. At low pH values there was an increase in furfural formation. Studies by Tatum et al. (1967, 1969) on non-enzymic browning of orange powders also showed that furfural was formed during acid-catalysed hydrolysis of vitamin C. It thus appears from the above that vitamin C is a likely candidate as the precursor of furfural in orange juice.

Dinsmore and Nagy (1972) have developed a test based upon the furfural content of juice to indicate deterioration during storage. In studies on single-strength orange juice (Nagy and Randall, 1973) and grapefruit juice (Nagy et al, 1972) furfural was found to be related to storage conditions and flavour change.

MATERIALS AND METHODS

Furfural Determination

The furfural content of orange juice was measured according to the method of Dinsmore and Nagy (1972).

Two hundred ml of single-strength orange juice was placed in a 1 l round-bottomed flask and distillation was carried out in an apparatus as described by Scott and Veldhuis (1966) (Fig. 35). The condenser was cooled by circulating cool water (18°C) through it, and previously chilled tubes, marked at 10 ml, were used for collecting the distillate. The heater control was adjusted to obtain a distillation rate of about 3 ml/min. Ten ml distillate was collected and used for furfural determination.

Preparation of reagent and measurement of colour

To prepare the 10% aniline reagent, 5 ml of freshly distilled aniline was diluted to 50 ml by volume with glacial acetic acid. To 2 ml of collected sample was added: 2 ml of ethyl alcohol and 1 ml of 10% aniline reagent at room temperature, and the intensity of colour was measured at 515 nm after 15-20 minutes.

Furfural standards

The furfural standard solution used was in the range of 10^{-3} to 10^{-6} molar. A calibration chart was obtained

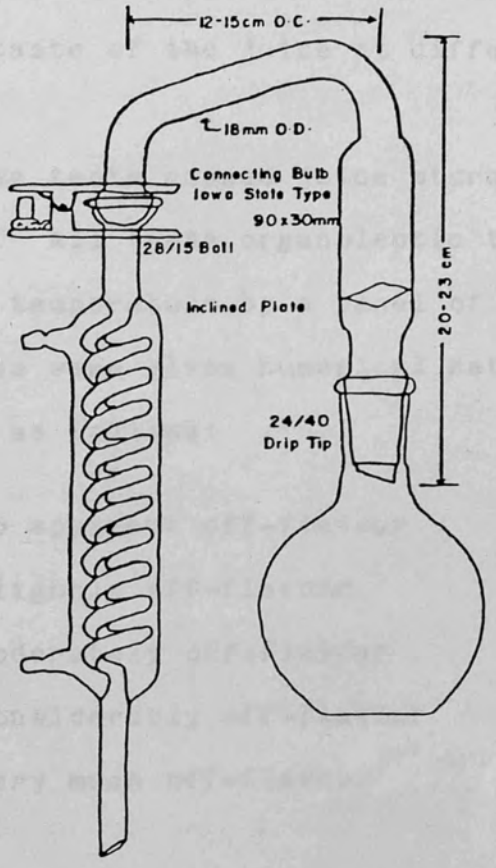


Fig. 35. Apparatus for distilling furfural

by plotting percentage transmittance against concentration of furfural. The furfural content of each orange juice sample was obtained from this chart.

Flavour Evaluation

Sensory evaluations were also carried out to determine changes in taste of the juice at different storage times.

In these tests canned juice stored at 5°C were used as controls. All these organoleptic tests were carried out at room temperature by a panel of tasters. These tasted juices were given numerical rating according to Curl (1947) as follows:

No apparent off-flavour	0
Slightly off-flavour	1
Moderately off-flavour	2
Considerably off-flavour	3
Very much off-flavour	4

RESULTS AND DISCUSSION

Effect of Canning

Content of vitamin C in fresh orange juice was measured as 50 mg/100 ml which decreased to 47 mg/100 ml after canning (Table 16), which was not significant. This corresponded to a retention of 95% of the vitamin C content of the original fresh juice. These results agreed with those of Moore *et al*. (1944) who showed that canning caused the retention of 97% of vitamin C in single-strength grapefruit juice.

Effect of canned Storage

Storage of canned orange juice at 7°C for 12 months resulted in a decrease in vitamin C from 47 to 44 mg/100 ml signifying a loss of 3.8 mg/100 ml (Table 17). This was not found to be significant and corresponded to 92% retention of vitamin C (Table 17 and Fig. 36). This implies that storing canned orange juice at 7°C for 12 months does not result in any significant loss in vitamin C content. Bissett and Berry (1975) found that SSOJ packaged in glass retained 87% of the initial vitamin C content after one year at 4.4°C. They also found that vitamin C retention was progressively lost at 10° and 15.6°C, being 84 and 79% respectively. Vitamin C retention at 26.7°C was reduced to 67% after eight months when their assay was discontinued because the juice was

Table 16. Vitamin C content and percentage retention during pasteurization and canning of single-strength orange juice compared with fresh samples

Sample	mg/100 ml	Percentage Retention
Fresh	48.8	100.0
Canned	47.4	95.0

Table 17. Effect of storage at two temperatures over a twelve months period on the vitamin C of canned single-strength orange juice. Graphical presentation of the data is shown in Fig. 36

Storage time (week, month)	mg/100 ml 7°C	% Retention 7°C	mg/100 ml 30°C	% Retention 30°C	Residual % loss 30°C
0	47.4 n.s.	100.0	47.4 p	100.0	0
1 week	47.2 n.s.	99.6	43.2 no	91.1	9
2 weeks	47.0 n.s.	99.1	42.0 mn	88.6	3
1 month	47.0 n.s.	99.1	40.3 lm	85.0	4
2 months	46.8 n.s.	98.7	38.5 kl	81.2	4
3 "	46.5 n.s.	98.1	36.6 k	77.2	5
4 "	46.2 n.s.	97.5	32.8 i	69.2	10
5 "	45.8 n.s.	96.6	29.2 gh	61.6	11
6 "	45.1 n.s.	95.1	26.4 efg	55.7	10
7 "	45.0 n.s.	94.9	25.2 ef	53.2	5
8 "	44.8 n.s.	94.5	24.0 de	50.1	5
9 "	44.6 n.s.	94.1	21.6 bcd	45.6	10
10 "	44.3 n.s.	93.4	20.5 abc	43.2	5
11 "	43.8 n.s.	92.4	19.4 ab	40.9	5
12 "	43.6 n.s.	92.0	18.3 a	38.6	6

n.s. = not significant

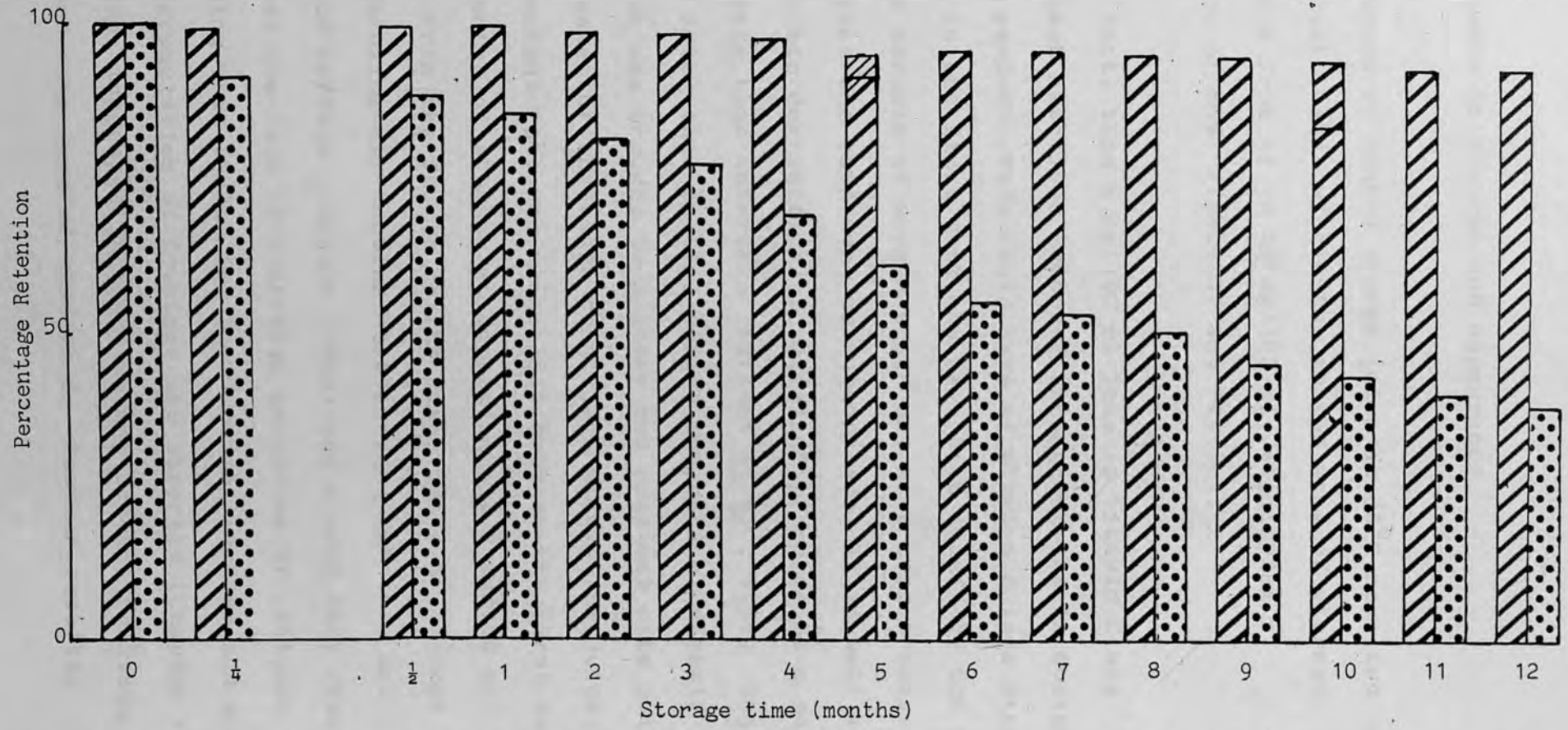


Fig. 36. Percentage retention of vitamin C of canned Sia-Varaz orange juice stored at 7° and 30°C over twelve months

unacceptable in flavour and appearance.

Storage of canned orange juice at 30°C resulted in significant losses within the second week of storage, decreasing from 47 to 42 mg/100 ml, signifying a retention of 88% (Table 17 and Fig.36).

In fact, this 5 mg/100 ml loss in vitamin C was the highest rate loss recorded throughout the 12 months storage period. This rapid loss of vitamin C (see Statistics p.274a) in the first week may have been caused by the presence of trace amounts of oxygen which may have been present in the juice since the cans were not deaerated. It is well known that aerobic degradation of vitamin C proceeds at a much faster rate than anaerobic (Kefford et al., 1959; Nagy and Smoot, 1977). After this initial period the rate of vitamin C breakdown was brought to a lower and constant rate owing to the onset of anaerobic conditions within the juice. This constant rate was maintained between the first and third months of storage at approximately 2 mg/100 ml/ month. From the fourth to the sixth month of storage this rate approximately doubled, but reasons for this are not known for certain. Huelin (1953) has stated that fructose increases the rate of anaerobic breakdown of vitamin C and during this period the hydrolysis of sucrose and a rapid accumulation of fructose was observed (Chapter VIII); therefore, increases in fructose may account for this increased rate loss of vitamin C. Another possible

explanation is that in the presence of even small amounts of oxygen vitamin C may react with it, catalysed by trace amounts of copper. The formation of H_2O_2 in this situation occurs. This catalytic effect of copper is enhanced by iron. The H_2O_2 formed during this reaction can lead to further oxidation of vitamin C (Weissberger and La Valle, 1944). In this study, breakdown of the lacquer was observed in some cans after four months of storage. On the other hand, the tin plate of the cans also has a beneficial influence by reacting preferentially with oxygen under acid conditions.

From the seventh month to the twelfth month of storage the rate of vitamin C loss was low and this remained fairly constant.

The total loss of vitamin C was 29 mg/100 ml over the total 12 months storage period at 30°C. This implicated a total loss of 61.4% of vitamin C. The results just presented are fairly similar to a number of other studies as follows: Sheft et al. (1949) have studied the effect of storage temperature on vitamin C content of canned SSOJ and have reported that the retention of vitamin C for 24 months storage were 95% and 50% at 10° and 26.5°C respectively. Freed et al. (1949) have also reported that canned SSOJ retained 80% and 5% vitamin C respectively when stored for 12 months at 22.2° and 37.8°C. More recently, Smoot and Nagy (1980) have reported that vitamin C

retention in canned single-strength grapefruit juice stored at 10, 20, 30, 40, and 50°C for three months were 99, 97, 90, 70, and 29% respectively.

Results in this study have indicated a constant build up of furfural, implying a deterioration in the quality of the juice.

Clegg (1964) has shown that vitamin C breakdown contributes to the darkening of the juice. As stated (Chapter VIII) the browning of the juice increased with storage time at the high temperature of storage and that free sugars were partly responsible for this by reacting with free amino acids in the Maillard reaction. It appears that vitamin C breakdown also plays an important part in producing this discolouration.

It may be said that at the 30°C storage temperature both the breakdown of vitamin C and the loss of free sugars are contributory to causing the darkening of the juice thereby reducing its acceptability.

The results of furfural determination of canned SSOJ at 7 and 30°C over 12 months storage are presented in Table 18. The furfural content of freshly processed orange juice was very low (5 µg/l) and this increased significantly at both storage temperatures over 12 months storage. At 7°C the concentration of furfural increased

Table 18. Furfural content of canned Sia-Varaz orange juice stored at 7° and 30°C over a twelve months period

Storage time (months)	Furfural content (µg/l)	
	7°C	30°C
0	5* a	5* a
2	12* ab	280 b
4	27 c	730 c
6	38 d	1300 d
8	50 e	1510 de
10	61 f	1850 f
12	73 g	2300 g

* Estimated

to 12 µg/l after two months storage, and this increase continued for the rest of the storage time reaching 73 µg/l by the twelfth month. The rate of increase doubled every two months until the fourth month, with a net increase of 7 and 15 µg/l respectively. After this initial rapid accumulation of furfural the rate of increase slowed down between the fourth and twelfth month.

At 30°C the furfural concentration increased to 280 µg/l after two months and reached 2300 µg/l after 12 months storage. This significant increase in furfural level after two months storage may be explained by the rapid loss of vitamin C of the 30°C juice (Table 17). A closer examination of the furfural level of the 30°C stored juice reveals that the furfural accumulation slowed down after six months' storage compared to the first six months of the experiment. This could be due to reduced levels of vitamin C in the 30°C stored juice after the first six months. Vitamin C content of the juice was also reduced to half of the original value after eight months storage (Table 17), and this loss of vitamin continued in the tenth and twelfth month. Nagy and Dinsmore (1974) have found that the vitamin C level of the orange juice had a direct effect on the furfural level in the stored canned SSOJ. The juice with the higher vitamin C concentration had the higher furfural level after storage at 5, 15, 21, and 35°C for 12 weeks.

Results of flavour evaluation of stored juices at 7 and 30°C are shown in Table 19. Flavour in canned orange juice was found to be well preserved by refrigerated storage. After a period of one year's storage at 7°C the juice was found by the taste panel to possess a distinct orange flavour, practically equal to that of the control and with no colour changes. Nagy and Randall (1972) have found that furfural values within the approximate values of 50-70 µg/l had a significant difference in flavour compared to the controls. In the present study, despite the fact that furfural levels were higher than those after ten and 12 months' storage at 7°C, no apparent off-flavour was detected. This may be explained by the findings of Dinsmore and Nagy (1972) that when furfural itself was added to a control orange juice at the 200 - 2000 µg/l, it was not recognizable by the taste panel. Further, the detection of flavour change depends very much upon the sensitivity of the taste panellists.

Monthly taste of the 30°C stored canned juice showed that after the first month's storage, the juice was distincly different from that stored at 7°C. This was rated as slightly off-flavour (1) (Table 19). In the present study, after two months' storage at 30°C, it was found that the flavour had deteriorated further (2). After four months' storage, the juice was off-colour owing to browning, and was considerably off-flavour (2+) owing to a caramelized and oxidized flavour. The flavour

Table 19. Flavour of canned stored single-strength orange juice stored at two temperatures over a twelve months period

Storage time (months)	Storage temperatures	
	7°C	30°C
0	0	0
2	0	2
4	0	2+
6	0	3
8	0	4
10	0	-
12	0	-

- = discontinued

taste conducted after six months' storage showed a flavour rating of (3). After eight months, this juice was disagreeable (4) and dark brown in colour (Fig. 31). No flavour evaluation was made after eight months' storage because of the definite change in colour and flavour. Higher storage temperature was not used in this study. However, Nagy and Dinsmore (1974) have recorded highly significant taste deterioration after only 2-4 days' storage of canned SSOJ at 35°C.

From the results of this study it can be seen that furfural level showed a progressive increase in juice with increasing storage time at the higher temperature. This led to earlier recognition of off-flavour and to earlier colourimetric detection of furfural compared to the lower temperature. However, furfural level may be regarded as an index of those substances possessing the off-odour of temperature-aged juice. Furthermore, furfural determination could be used as a useful and sensitive tool in revealing the onset of deterioration that will cause off-flavour.

CONCLUSION

An examination of Table 17 shows that this investigation has brought out a number of points worthy of note, some of which are apparent and others which may be interpreted only by the aid of additional information. Of primary importance is the fact that low temperature is essential if adequate amounts of vitamin C are to be retained during storage. In contrast, the higher storage temperature (30°C) caused a rapid loss of vitamin C in a comparatively short time. Moreover, the results have indicated that the vitamin C loss during long storage periods was mainly due to anaerobic reactions; total vitamin loss during that period exceeding total loss due to oxidative reactions.

An average glass of orange juice (177 ml) from the canned SSOJ stored at 7°C for 12 months contains 77 mg vitamin C which is slightly more than the RDA recommended by FDA (1973) of 60 mg. Furthermore, the flavour is unchanged. Therefore, a glass of SSOJ stored at 7°C for a year may provide all the vitamin C required by an adult.

One glass of orange juice stored at 30°C for 12 months does not fulfil the minimum RDA while two glasses would give more than the minimum if it could be consumed at all as the juice would by then be completely off-flavour.

Considering the overall results of the juice kept at 30°C it was observed that up to three to four months' storage, a glass of this juice may be used as a sole source of vitamin C to fulfil the RDA, but after this period supplementary vitamin C would be needed to satisfy this demand.

A coincidental fact is that flavour impairment was noticed after approximately four months' storage at 30°C. Therefore, after that time, canned single-strength Sia-Varaz orange juice is not only regarded to be comparatively low in vitamin C but is also very poor in its quality as judged by its taste.

CHAPTER X

EFFECT OF CANNING AND CANNED STORAGE ON FREE AMINO ACIDS

INTRODUCTION

The nitrogenous compounds in citrus represent a small but important group of substances. Smith (1963) has calculated that the fruit contains about 25% of the total 'N' of the orange tree. Roughly 50% of the 'N' absorbed by the tree each year end up in the fruit. The largest fraction within the nitrogenous group is the free amino acids, which typically account for about 70% of the 'N' in various citrus juices.

As was discussed earlier, the composition of orange juice is dependent on a number of factors, namely, climate, root stock, light, soil texture, fertilizer, and age of the parent tree. Processing is another source of variation in the nitrogenous constituents of citrus juices. Factors such as processing conditions used, temperatures of storage and time of storage would play a part in affecting the nutritional status of the food. The colour of the juice is also a primary factor in determining the acceptance of the product but it is unclear at present the exact role amino acids play in affecting this quality.

The main colour problem facing the canned citrus industry is the non-enzymic browning occurring during storage. Acidity, temperature, presence or absence of oxygen and nature of the container are important factors affecting the rate and type of browning. A number of reports indicate that more than one type of reaction may be involved in this browning (Blundstone et al., 1971). 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 acidity of the latter. Studies by Huffman (1974) on the other hand, have shown that the amino acids and sugars are more than just of minor importance in the darkening of citrus juices.

Huffman (1974) has shown that orange juice treated with cationic ion-exchange resins to remove amino acids, when heated for 20 minutes at 100°C, and then stored at 20°C, were less subject to browning than were their respective untreated controls. On the other hand, Townsley et al. (1953) have stored centrifuged protein-free single-strength orange juice for four months at 37°C and have reported that the protein-free juice darkened about three times faster than whole juice. This would suggest that browning inhibitors possibly were present in the insoluble orange juice solids, and that free amino acids present in the solution increase the browning reaction.

Ismail and Wolford (1967) have shown that after pasteurization there were no major qualitative differences in composition of the amino acids of the canned SSOJ.

Studies by Joslyn (1957) on Valencia oranges have shown that after four years of storage at room temperature of fresh orange juice to which sodium benzoate had been added, lysine apparently disappeared and glutamic acid decreased. Alanine, arginine, asparagine and proline were not affected appreciably. These results indicate that of the amino acids present in Valencia orange juice, lysine and glutamic acid are apparently more reactive.

During processing and storage of orange juice, Rockland (1961) found a 60% increase in amino nitrogen within 60 seconds when the juice was heated at 93°C. These values were decreased to a level slightly higher than that of fresh juice when it was stored for five months at 38°C.

A strong relationship exists between ascorbic acid, sugars and amino acids which are implicated in browning (Joslyn, 1957). Amino acids are said to inhibit browning in the initial stages but in later stages increase it (Adrian, 1982).

This investigation was designed to study the effect of canning and canned storage at 7° and 30°C on the free amino acids of single-strength orange juice from the Sia-Varaz variety.

Methods used for the determination of free amino acids were as those already described on p.87.

RESULTS AND DISCUSSION

Effect of Canning

The canning process has brought about an increase in free amino acids (FAA) from 520.5 to 1007.8 mg/100 ml (Table 20). This corresponds to an increase of 193.6% of FAA in orange juice. The increase was greater than that found by Rockland (1961). Although the canning of orange juice has increased the amino acid status of the product there was no qualitative change in the individual amino acids. The cause of this increase could be due to breakdown of lipo-proteins in the cloud. As already mentioned on p. 179, the cloud consists mainly of membraneous material and pectin. The juice was subjected to high temperature during pasteurization which seems to have disrupted some of the protein within the membraneous material, causing amino acids (AA) to be released into the aqueous portion of the juice.

Table 20 and Fig. 37 show that histidine and methionine decreased whereas tyrosine remained fairly stable. Substantial increases were noted in all remaining AA. However, the results, if expressed as a percentage of total AA (Table 20), display a different picture. Histidine, arginine, asparagine, threonine, serine and methionine all showed decreases, while NH_3, glutamic acid and glycine remained constant. Cysteine, found as a trace

Table 20. Effect ot canning on free amino acids of fresh Sia-Varaz orange juice. Graphical presentation of the data is shown in Fig. 37

	Free amino acids			
	Fresh mg/100 ml	Canned	Fresh % of total	Canned
Lys	10.6	27.0*	2.0	2.7
His	2.0	0.5*	0.4	0.0
NH_3	12.7	22.9*	2.4	2.3
Arg	122.6	212.6*	23.5	21.1
Asp	50.3	60.7*	9.7	6.0
Thr	61.7	68.3*	11.8	6.8
Ser	40.0	53.8*	7.7	5.3
Glu	35.6	70.5*	6.8	7.0
Pro	133.9	297.2*	25.7	29.5
Gly	3.0	6.8*	0.6	0.7
Ala	26.1	96.1*	5.0	9.5
Cys	T	0.6*	T	0.0
Val	6.3	30.6*	1.2	3.0
Met	0.5	0.3*	0.0	0.0
Ileu	3.2	28.8*	0.6	2.8
Leu	3.0	10.5*	0.6	1.0
Tyr	2.5	3.0*	0.5	0.0
Phe	6.0	17.6*	1.1	1.7
Total	520.5	1007.8*	99.6	99.4

* indicating significant differences at 5% level

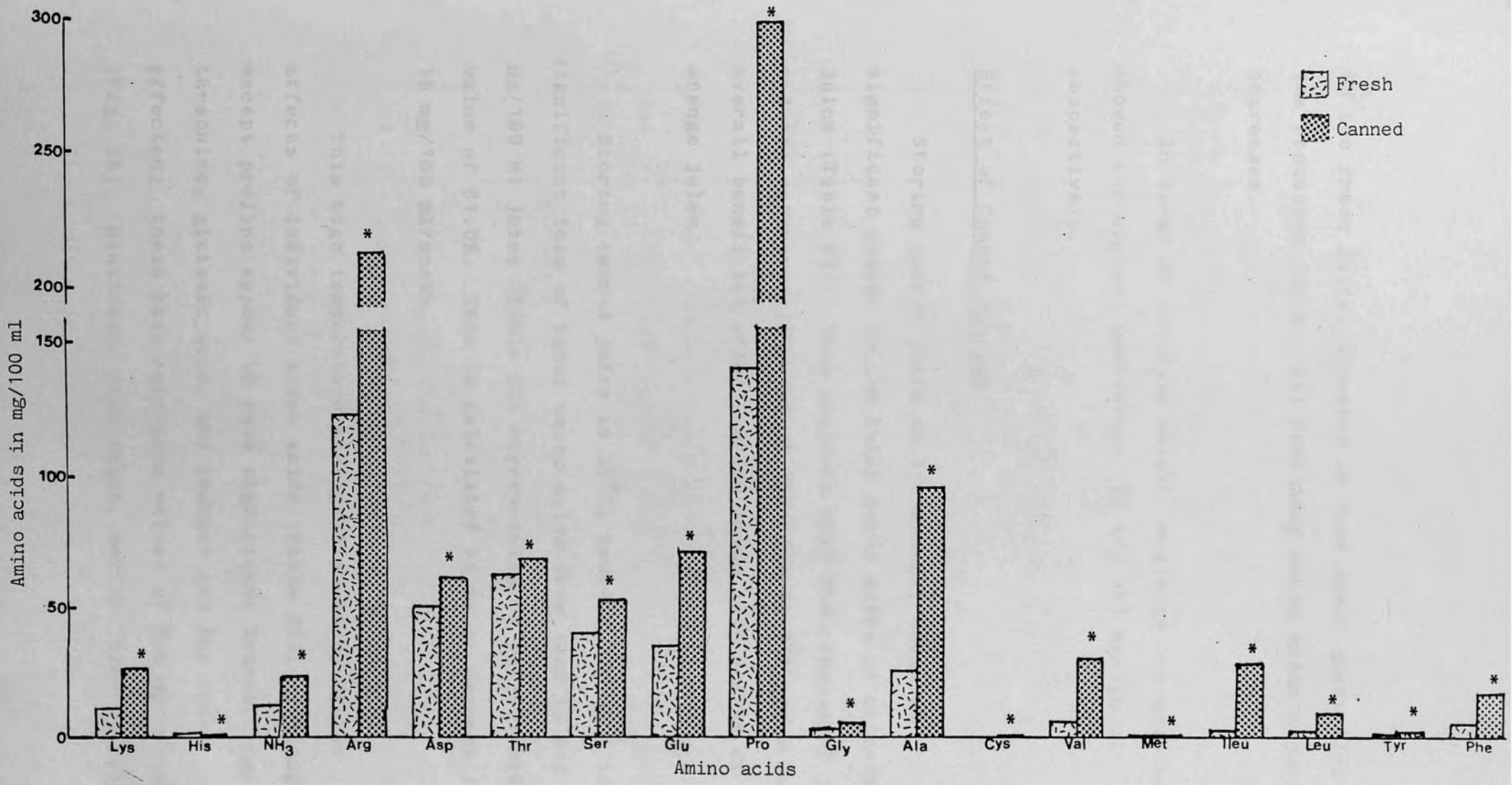


Fig. 37. The effect of canning on free amino acids of fresh Sia-Varaz single-strength orange juice

in the fresh juice, appeared in very minor quantities in the processed juice. All remaining amino acids showed increases.

In terms of absolute weight, arginine and proline showed the highest increases: 90 and 163 mg/100 ml respectively.

Effect of Canned Storage

Storing canned juice at 7°C brought about no significant change in the total amino acids of processed juice (Table 21). This suggests that the canning of juice and the subsequent storing of the cans at 7°C gives an overall beneficial effect to the amino acid status of the orange juice.

Storing canned juice at 30°C, however, results in a significant loss of total amino acids from 1008 to 817 mg/100 ml juice (Table 22) representing an overall retention value of 81.0%. This is calculated to be an average loss of 16 mg/100 ml/month.

This high temperature storage also shows varied effects of individual amino acids (Table 22). Although all except proline appear to show significant losses, arginine, threonine, glutamic acid, and leucine are the least affected; these have retention values of 80% or above (Fig. 38). Histidine, asparagine, serine, alanine, valine,

Table 21. Effect of storage at 7°C over a twelve months period on the free amino acids of canned single-strength orange juice. Values are expressed as mg/100 ml

Amino acids	Storage time (months)						
	0	2	4	6	8	10	12
Lys	27.0	27.2	26.9	27.8	26.0	25.8	26.1
His	0.5	0.5	0.5	0.4	0.5	0.5	0.5
NH_3	22.9	21.8	22.6	22.0	21.0	22.3	22.0
Arg	212.6	207.3	215.0	218.9	210.2	220.0	216.8
Asp	60.7	57.2	58.0	56.9	55.3	62.0	61.7
Thr	68.3	70.1	68.0	69.6	65.3	66.0	71.1
Ser	53.8	53.0	56.8	50.2	52.9	51.0	54.6
Glu	70.5	72.8	69.1	73.0	74.2	72.5	74.0
Pro	297.2	290.0	298.2	297.0	299.0	286.7	301.3
Gly	6.8	6.5	6.1	5.2	5.9	7.2	6.0
Ala	96.1	90.0	100.1	102.5	92.0	103.1	94.7
Cys	0.6	0.6	0.6	0.5	0.6	0.6	0.6
Val	30.6	30.8	31.9	28.7	29.0	30.0	31.1
Met	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Ileu	28.8	27.2	29.1	28.0	27.0	30.2	30.0
Leu	10.5	10.1	9.8	9.5	11.0	10.3	10.0
Tyr	3.0	3.0	3.1	3.0	2.9	3.0	3.1
Phe	17.6	17.9	16.8	18.2	17.0	18.5	18.0
Total	1007.8	986.3	1012.9	1011.7	990.0	1100.0	1021.9

Table 22. Effect of storage at 30°C over a twelve months period on the free amino acids of canned single-strength orange juice. Values are expressed as mg/100 ml. Graphical presentation of the data is shown in Fig. 38

Amino acids	Storage time (months)						
	0	2	4	6	8	10	12
Lys	27.0 g	20.6 f	16.3 e	12.1 cd	9.8 bc	7.3 ab	6.9 a
His	0.5	0.6	0.4	0.5	0.5	0.5	0.3
NH_3	22.9 a	25.4 ab	29.6 bcd	27.8 bc	30.5 bcde	36.0 ef	37.9 fg
Arg	212.6 bcdefg	202.4 bcdef	208.0 bcde	200.0 abcd	197.4 abc	180.2 ab	174.9 a
Asp	60.7 efg	58.6 ef	55.4 cde	54.0 cd	49.5 abc	45.1 ab	44.0 a
Thr	68.3 cdefg	66.1 cdef	65.3 cde	64.7 cd	62.1 abc	52.0 a	54.6 ab
Ser	53.8 efg	55.2 def	50.7 de	48.9 cd	42.0 ab	43.1 abc	41.3 a
Glu	70.5 bcdefg	69.1 abcdef	67.0 abcd	67.6 abcde	64.2 abc	60.5 a	61.8 ab
Pro	297.2 n.s.	293.3 n.s.	287.0 n.s.	273.4 n.s.	268.5 n.s.	266.1 n.s.	257.2 n.s.
Gly	6.8 g	5.2 ef	4.7 cde	4.4 bcd	4.0 abc	3.7 ab	3.5 a
Ala	96.1 fg	85.1 abcdef	81.0 abcde	77.2 abcd	75.6 abc	75.1 ab	74.6 a
Cys	0.6 cd	0.7 de	0.6 cd	0.5 bc	0.6 cd	0.4 b	0.2 a
Val	30.6 efg	31.7 ef	29.5 de	26.6 cd	25.0 bc	21.3 ab	19.4 a
Met	0.3 b	0.3 b	0.2 a	0.4 c	0.3 b	0.3 b	0.2 a
Ileu	28.8 defg	27.1 cdef	25.0 cde	26.2 cd	24.7 bc	21.6 ab	20.5 a
Leu	10.5 abcdef	9.8 abcd	9.5 abc	11.0 bcdefg	10.0 abcde	9.2 ab	8.5 a
Tyr	3.0 defg	2.9 cdef	2.7 bcde	2.5 abc	2.6 abcd	2.4 ab	2.2 a
Phe	17.6 g	14.3 cde	14.2 cd	15.1 cdef	13.3 bc	10.2 ab	8.6 a
Total	1007.8	968.4	947.1	913.0	880.6	835.0	816.5

n.s. = not significant

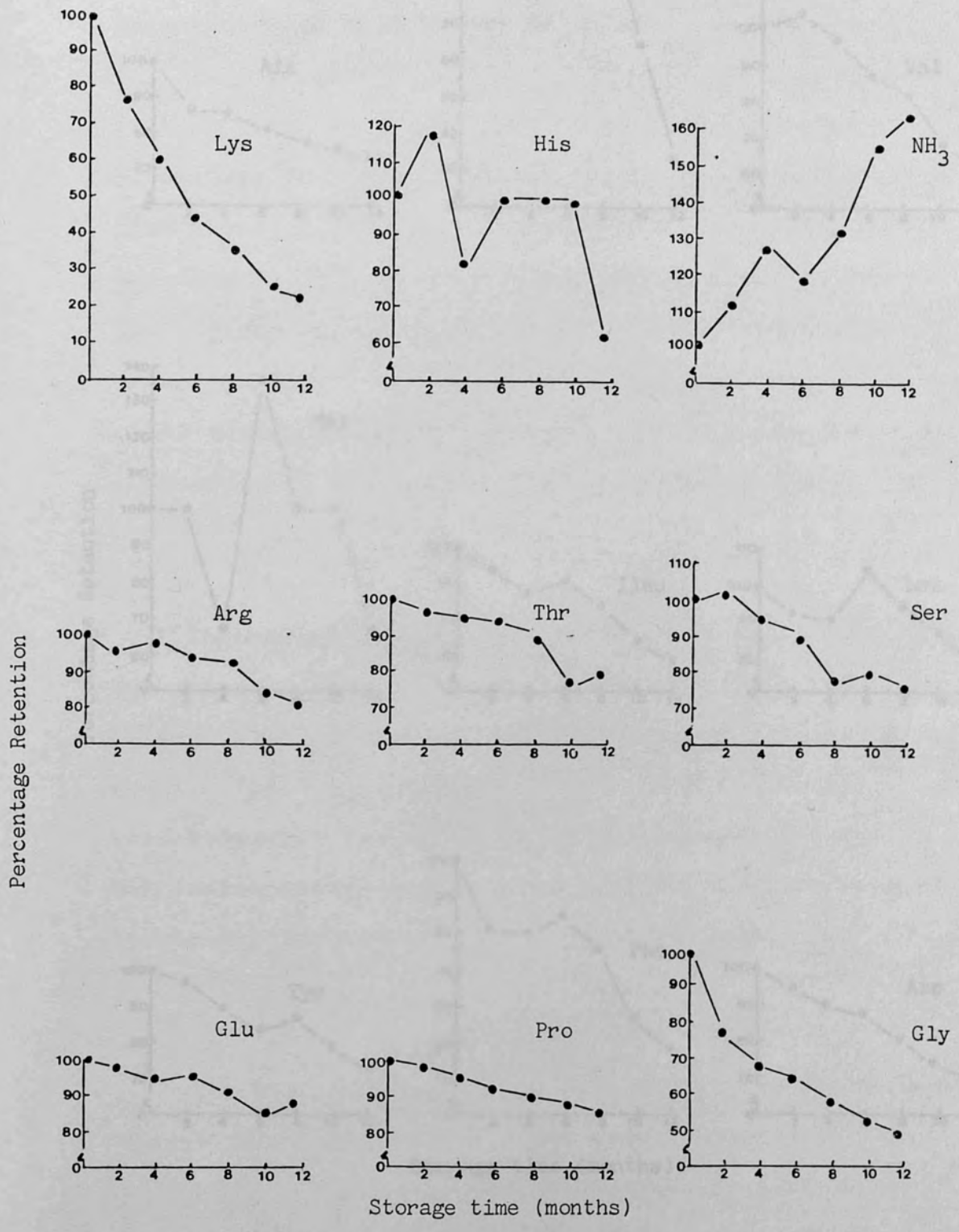


Fig. 38. Percentage retention of free amino acids of canned Sia-Varaz orange juice stored at 30°C over a twelve months period. For significant differences refer to Table 22

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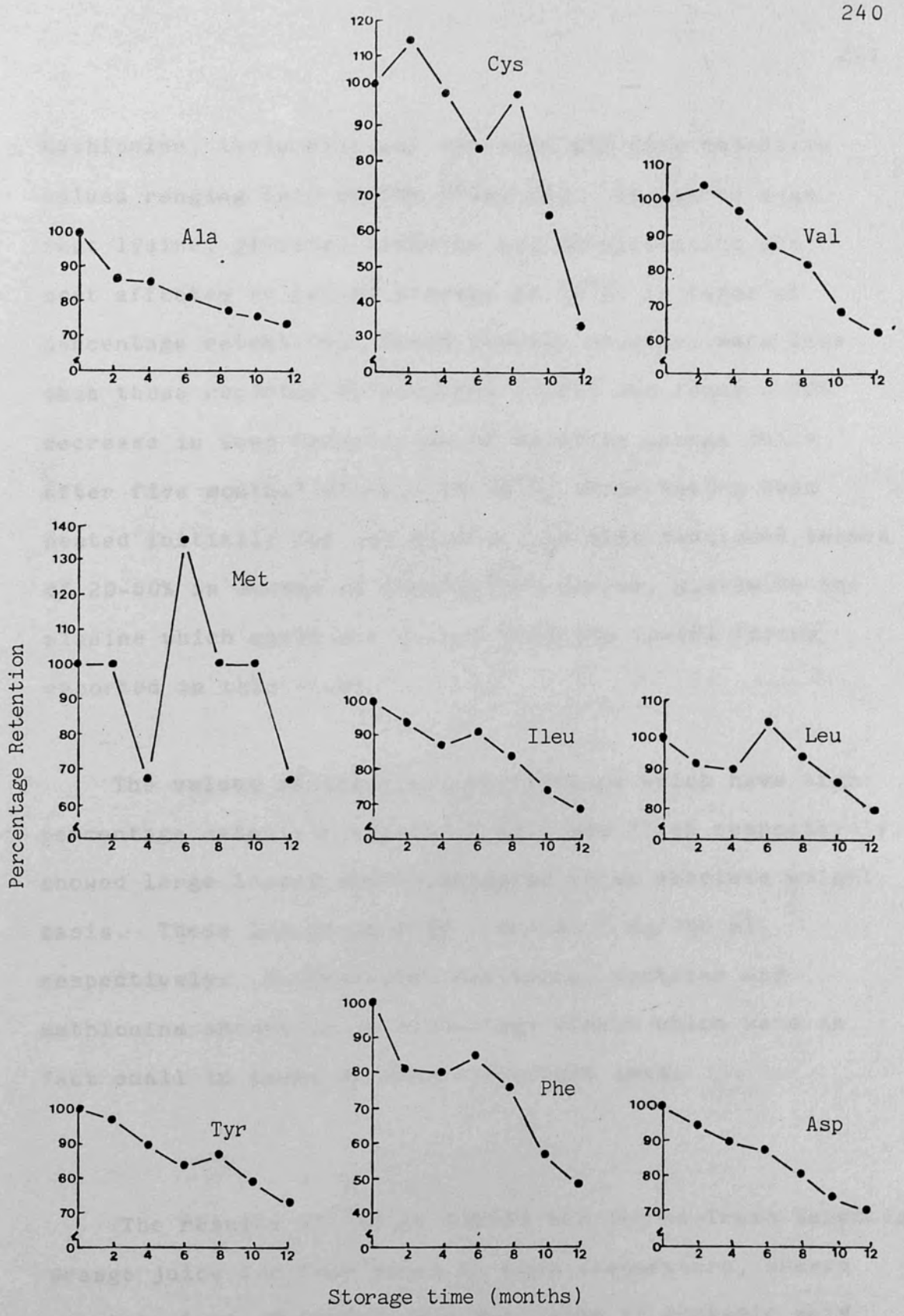

Ala
Cys
Val
Met
Ileu
Leu
Tyr
Phe
Asp
Percentage Retention
Storage time (months)

Fig. 38 (contd)

methionine, isoleucine and tyrosine all have retention values ranging from 60-80% (Fig. 38). It can be seen that lysine, glycine, cysteine and phenylalanine are most affected by canned storage at 30°C, in terms of percentage retention. These losses, however, were less than those reported by Rockland (1961) who found a 70% decrease in free amino acids of Valencia orange juice after five months' storage at 38°C, after having been heated initially for one minute. He also mentioned losses of 20-60% in levels of asparagine, serine, glutamine and alanine which again are higher than the 12-28% losses reported in this study.

The values of arginine, and alanine which have high percentage retention values of 82.3 and 77.6% respectively, showed large losses when considered on an absolute weight basis. These losses were 37 and 21 mg/100 ml respectively. Furthermore, histidine, cysteine and methionine showed large percentage losses which were in fact small in terms of absolute weight loss.

The results of Joslyn (1957) who stored fresh Valencia orange juice for four years at room temperature, showed complete loss of lysine, and decreases in glutamic acid are to some extent paralleled in the present study where the amount of lysine and glutamic acid loss was large. The fact that Joslyn (1957) found that alanine, arginine,

asparagine and proline were not appreciably affected shows that canned storage at elevated temperatures has no adverse affect on these particular amino acids.

On the whole, 12 of the 17 identified amino acids showed losses of less than 15 mg/100 ml canned juice. The remaining five amino acids, namely lysine, arginine, asparagine, proline and alanine cumulatively showed a total loss of 136 mg/100 ml after 12 months of storage at 30^{o}C. This represents 70% of the loss of total amino acids. Wolfrom et al. (1974) was of the opinion that arginine was relatively abundant in Valencia orange juice and hence considered that it may play an important role in the deterioration of orange juice on storage. Non-enzymic browning was implicated as a source of loss as it was shown that arginine gave more colour on browning than lysine. Results in the present study show that losses in arginine were almost twice as high as those of lysine and supports the above suggestion.

Wolfrom et al. (1974) have studied model systems of the browning reaction involving glucose and various amino acids. They found that the order of most reactive to least reactive, in terms of browning, was arginine, asparagine, lysine, alanine and proline. Since there were appreciable levels of free sugars and vitamin C in freshly canned orange juice which were then observed to decrease substantially after storage for 12 months, suggests that a

certain amount of this loss could be due to browning reactions. This, combined with the observation that appreciable amounts of amino acids also disappeared, implies that the sugars or vitamin C may have reacted with the free AA in the Maillard reaction to form brown pigments.

The loss in the amino acids is higher than that in ascorbic acid (Chapter IX) and this again supports the suggestion that amino acids play more than just a minor part in the browning reaction. Adrian (1982) has mentioned that the intensity of the Maillard reaction generally increases with the elevation of the pH from 3 to 9. So at pH 3.8 (pH of orange juice in the present study), there are reactions between sugars and amino acids, contributing to the browning reaction. The canned orange juice was found to possess a pH of 3.8 which makes it possible for amino acid-sugar reactions. It is also possible that some of the amino acids may have been lost through the increase in storage temperature and therefore not all amino acids would have taken part in Maillard reactions.

In general, the present study supports the observation of Huffman (1974) who pointed out that amino acids played an important role in the browning of orange juice. However, they are not the only compounds involved in the browning reaction. Various other compounds such as vitamin C and

sugars may also take part in the browning of the juice, as already discussed.

CHAPTER XI

EFFECT OF CANNING AND CANNED STORAGE ON LIPIDS

INTRODUCTION

Two important characteristics of citrus juice, flavour and colour, are related organoleptically as well as physiochemically. The visual significance of colour in influencing the flavour evaluation of orange juice was revealed by a consumer survey which indicated that colour has a profound effect on consumer preference (CECO, 1965). Colour is also physiochemically related to flavour through the suspended particulate matter in fresh orange juice commonly referred to as 'cloud'. Loss of cloud results in an almost colourless, sour-sweet liquid of poor acceptance and little commercial value (Mizrahi and Berk, 1970).

Many investigations on cloud loss have ascribed the problem to the enzymatic degradation of pectic substances. The stability of the suspension is enhanced by heat treatment, which inactivates pectinesterase. This enzyme initiates a series of reactions producing insoluble pectates which settle out, finally resulting in juice

clarification. Pectinesterase activity is sharply reduced by temperatures of 49° to 65°C and even further reduced to a very low activity by heating at 88°C (Bissett et al., 1953).

The physical characteristics of the cloud have been the subject of many studies. Mizrahi and Berk (1970) have suggested that oil droplets closely associated with the surface of the cloud particles exert a stabilising effect on the suspension by decreasing the average density of the particles and attaining a density similar to that of the serum. The droplets of oil account for the rather high lipid content of the cloud compared to the lipid-deficient structural tissues of the fruit (Scott et al., 1965).

These observations add further support to those of Curl (1947) who found in his storage studies that the flavour deterioration was less in the filtered than in unfiltered orange juice. The suspended material had been removed from the filtered juice and Curl and Veldhuis (1947) concluded that this material, probably the lipid, must play an important part in the off-flavour which develops on storage.

Processed citrus juice is highly susceptible to off-flavour and off-odour development when stored at adverse temperatures and for prolonged storage period (Swift and Veldhuis, 1951). Early studies by Nolte and von Loesecke

(1940) on fresh and canned Florida Valencia orange juice have shown that temperature aged juice differed from fresh juice by increased saponification and peroxide values and by the presence of carboxyl compounds. The implied importance of lipids in off-flavour development stimulated further investigations of citrus lipids.

It may be argued that lipids are only a minor component of the juice, hence any changes in composition would have a negligible effect on juice flavour. This does not detract from their potential importance in off-flavour development as inferred from results published to date. There is evidence that another striking difference in fresh and aged Valencia juice was a decrease in phospholipids to 10% of their original value, nitrogen containing lipids to 20% and complete disappearance of phosphatidyl choline (Huskins et al., 1952). Commercial orange juice held in storage at elevated temperatures were also found to accumulate unesterified and unsaturated fatty acids.

Degradation products derived from the oxidation of unsaturated fatty acids have been implicated as one of the causative factors in citrus off-flavour development (Huskins and Swift, 1952). These observed changes in citrus lipids follow the general pattern of partial or complete hydrolysis to fatty acids and other components followed by oxidation of unsaturated fatty acids as proposed for orange juice.

The phospholipids in juice are highly susceptible to enzymatic breakdown. Enzymes which may be involved in the formation of free fatty acids during the storage of juice include lipases, steryl esterases and phospholipases which hydrolyse the glycerides, sterol esters and phospholipids respectively (Kates, 1960).

Together with other individual lipid components, the fatty acids have been studied in depth in the present study, in order to correlate any relationship between quantitative changes of constituents and deterioration in quality of stored canned Sia-Varaz orange juice. This will now be discussed.

Methods used for the determination of lipids were as those already described on p.117.

RESULTS AND DISCUSSION

Effect of Canning on Total Lipids

There was a significant increase in total lipids of orange juice after canning from 1212 to 1302 mg/100 g powder (Table 23).

Effect of Canning on Simple Lipids

There was an increase in simple lipids from 315 mg/100 g powder in fresh juice to 321 mg/100 g powder in canned juice (Table 23). This, however, was found not to be significant, hence it was concluded that canning of Sia-Varaz orange juice had no significant effect on the

Table 23. Effect of canning on the lipids of fresh Sia-Varaz orange juice. Graphical presentation of the data is shown in Fig. 39

Lipids (mg/100 g powder		
	Fresh	Canned
Simple lipids		
MG	6.5	6.5
DG	19.1	20.3
FS	80.0	83.3
UNK	1.0	1.1
FFA	33.8	36.5
FG	47.2	47.6
SE	74.5	73.6
HC	53.0	52.6
Total simple lipids	315.1	321.5
% of total simple lipids	26.0	24.7
Phospholipids		
PE	25.6	288.8*
PC	207.3	245.9*
PI	51.3	45.9*
PS	34.9	37.1
PA	47.4	63.7*
PG	28.3	20.7*
LPC	19.7	25.2*
UNK	13.2	13.3
Total phospholipids	658.1	740.6*
Glycolipids		
MGDG	140.3	142.7
CMH	98.1	96.8
Total glycolipids	238.4	239.5

/continued...

Table 23 (contd.)

Lipids (mg/100 g powder)		
	Fresh	Canned
Total complex lipids	896.5	980.1*
Total lipids	1211.6	1301.6
Complex lipids % of total lipid	74.0	75.3
Phospholipid % of complex lipid	73.4	75.6
Glycolipid % of complex lipid	26.6	24.4

* indicating significant differences at 5% level

simple lipid content. It was also observed that the individual constituents showed no significant change (Table 23 and Fig. 39).

There was no significant change in total free fatty acid content after canning (Table 23 and Fig. 39). Palmitic (C16:0), C18:1, C18:2 and C18:3 fatty acids were identified but no compositional changes noted.

Effect of Canning on Complex Lipids

There was a significant increase in total complex lipids from 896 mg/100 g powder before canning to 980 mg/100 g powder (Table 23). This gave a net increase of 9.3%. The glycolipids did not appear to be affected to any extent (Table 23 and Fig. 39).

Among the phospholipids, substantial increases were noted in PC from 207 to 246 mg/100 g powder and PE from 256 to 289 mg/100 g powder corresponding to net increases of 19 and 13% respectively (Table 23 and Fig. 39). A different picture, however, emerged if the results were expressed on a percentage increase basis where LPC and PA showed the maximum increases of 28 and 34% respectively. Phosphatidyl glycerol showed a substantial loss of 27% after canning. The likely explanation for the increases is that the high temperature treatment during pasteurization may have caused disruption of the membrane structure further (in cloud), hence

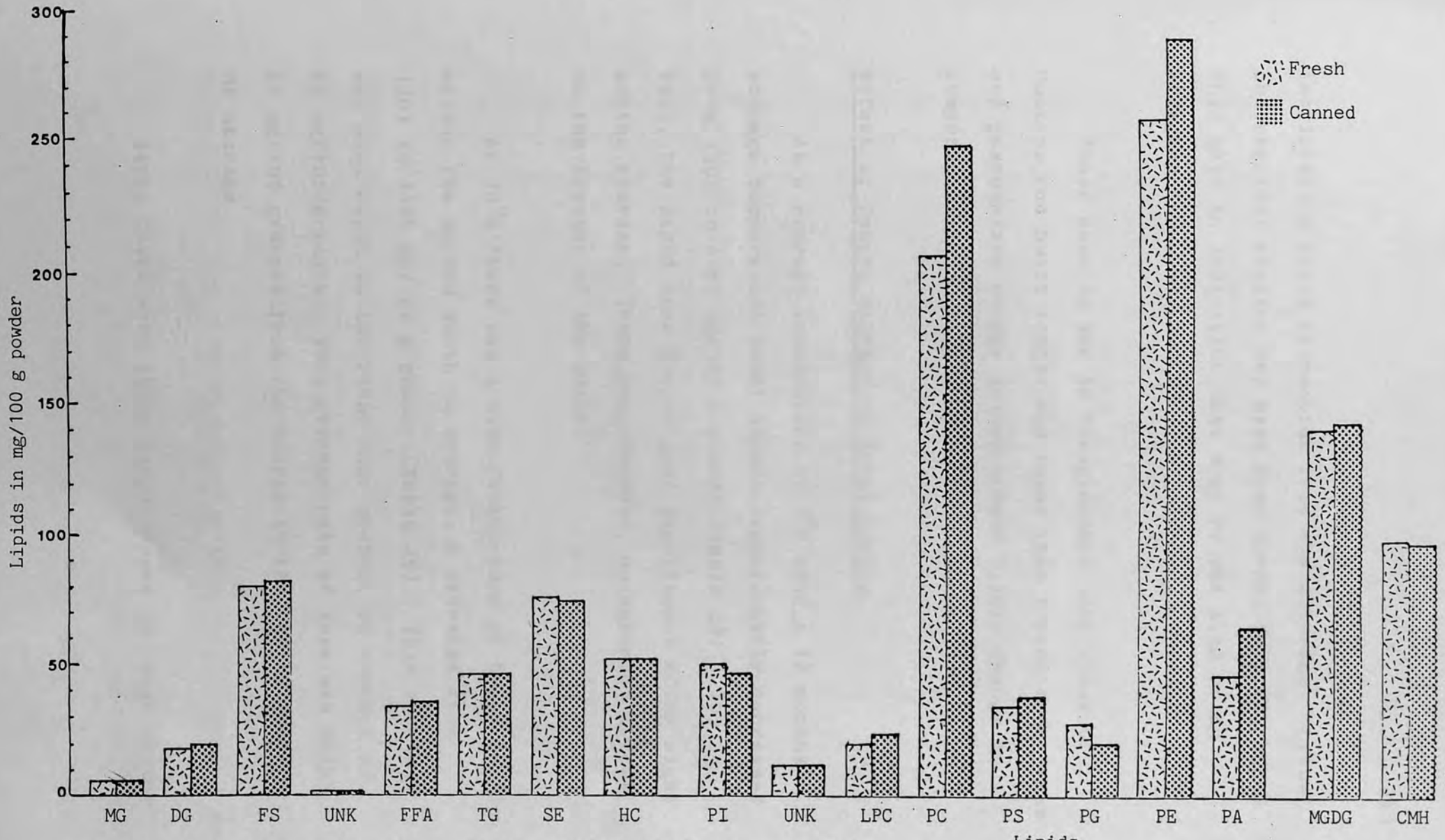


Fig. 39. The effect of canning on lipids of fresh Sia-Varaz orange juice.

facilitating lipid extraction from the membrane. Lyso-phosphatidyl choline may have been formed from PC, hence this gave an indication that some PC was also being affected.

These results are in disagreement with those of Huskins and Swift (1953) who found that lipids from fresh and pasteurized orange juices showed little change in composition.

Effect of Canned Storage on Total Lipids

At a storage temperature of 7°C over a 12 months' storage temperature total lipids significantly decreased from 1302 to 1187 mg/100 g powder (Table 24). In fact, the lipid loss became just significant after eight months storage. There was, however, no apparent effect on the flavour of the juice.

At 30°C there was a significant loss of total lipids within the second month of storage, a decrease from 1301 to 1145 mg/100 g powder (Table 25). This decrease was most rapid in the first four months, an average of 67 mg/100 g/month. This average rate of loss was only 21 mg/100 g/month from the fourth to the twelfth month of storage.

Since there were these large losses in total lipids

Table 24. Effect of storage at 7°C over a twelve months storage period on the simple lipids of canned single-strength orange juice. Graphical presentation of the data is shown in Fig. 40

Storage time (months)	Simple lipids (mg/100 g powder)								Total simple lipids (mg/100 g powder)	Simple % of total lipid	Total lipid (mg/100 g powder)	% increase of FFA	Total simple lipids - FFA (mg/100 g powder)
	MG	DG	FS	UK	FFA	TG	SE	HC					
0	6.5 bcd	20.3 def	83.3 n.s.	1.1 b	36.5 a	47.6 n.s.	73.6 n.s.	52.6 n.s.	321.5 a	24.7	1301.6	0	285.0
2	6.5 bcd	19.0 bcde	82.0 n.s.	1.1 b	42.6 ab	46.9 n.s.	72.9 n.s.	52.0 n.s.	323.0 ab	25.2	1282.9	16.7	280.4
4	6.5 bcd	18.3 abcd	79.3 n.s.	1.1 b	52.6 c	45.6 n.s.	70.8 n.s.	52.0 n.s.	326.2 abc	25.8	1264.9	44.1	273.6
6	5.2 abc	16.3 abc	77.3 n.s.	1.1 b	67.4 d	44.3 n.s.	69.5 n.s.	51.3 n.s.	332.4 abcd	26.5	1254.9	84.6	265.0
8	4.5 ab	15.6 ab	76.6 n.s.	0.5 a	81.4 e	43.6 n.s.	67.5 n.s.	50.0 n.s.	339.7 abcde	27.6	1231.5	123.0	258.0
10	3.2 a	15.6 ab	73.3 n.s.	-	92.8 f	42.2 n.s.	66.8 n.s.	50.0 n.s.	343.9 bcdef	28.4	1211.3	154.2	251.0
12	3.2 a	15.0 a	73.9 n.s.	-	96.2 fg	41.6 n.s.	65.5 n.s.	48.6 n.s.	344.0 bcdefg	29.0	1187.4	163.6	248.0

n.s. = not significant

Table 25. Effect of storage at 30°C over a twelve months period on the simple lipids of canned single-strength orange juice. Graphical presentation of the data is shown in Fig. 40

Storage time (months)	Simple lipids (mg/100 g powder)								Total simple lipids (mg/100 g powder)	Simple % of total lipid	Total lipid (mg/100 g powder)	% increase of FFA	Total simple lipids - FFA (mg/100 g powder)
	MG	DG	FS	UK	FFA	TG	SE	HC					
0	6.5 de	20.3 def	83.3 defg	1.1 b	36.5 a	47.6 cdefg	73.6 cdefg	52.6 cdef	321.5	24.7	1301.6	0	285.0
2	6.5 de	20.3 def	81.3 def	1.1 b	98.8 b	45.6 cdef	71.6 cdef	52.6 cdef	377.8	33.0	1145.4	170.7	279.0
4	5.2 cd	18.3 cde	74.6 abcde	0.5 a	166.5 c	44.3 bcde	66.9 cde	50.6 cde	426.9	41.4	1030.0	356.2	260.0
6	3.9 abc	17.7 cd	71.9 abcd	0.5 a	190.6 d	42.9 bcd	64.2 abcd	47.3 abcd	439.0	46.2	950.2	422.1	265.0
8	3.9 abc	16.3 bc	67.9 abc	-	220.1 e	40.9 bc	61.5 abc	45.3 abc	455.9	49.8	915.2	503.0	236.0
10	2.5 ab	14.3 b	65.9 ab	-	240.2 f	38.2 ab	56.8 ab	43.3 ab	461.2	52.7	875.1	558.1	221.0
12	1.8 a	11.0 a	62.5 a	-	268.4 g	33.5 a	53.5 a	40.6 a	471.3	54.8	859.7	635.3	203.0

in orange juice it was worth taking a closer look at the individual lipid classes, which will be discussed now.

Effect of Canned Storage on Simple Lipids

At 7°C storage temperature the decrease in the individual simple lipids followed a uniform pattern over the 12 months storage period (Table 24). However, these decreases appeared to be significant only after eight months of storage, implying that a certain amount of deterioration may have taken place then. On regular testing of the flavour, however, no deterioration in taste was evident. This suggests that oxidative changes in the lipid molecules stored at 7°C for 12 months were very low. This was despite the fact that FFA content increased from 36 to 96 mg/100 g powder, indicating an almost three-fold increase. These results support the suggestion of Nagy and Nordby (1970) that high carbon number fatty acids as found in orange juice, contribute very little to flavour. Their importance, however, is as precursors to many volatile off-flavour compounds. Fatty acid oxidative products, formed by autocatalytic and enzymic mechanisms, have also been implicated in off-flavour development in aged orange juice by Huskins and Swift (1953).

There seems to be a pronounced effect on simple lipids due to long term storage at 30°C. There was a significant increase from 321 to 471 mg/100 g powder after 12 months (Table 25). At first the percentage of simple

lipids to total lipids was 24.7% (Table 25) whereas after 12 months of storage, this value increased to 54.8% of total lipids.

These observations, however, give a misleading picture from the standpoint of the majority of the simple lipid individuals since this increase was solely due to a large increase in free fatty acids. In order to gain a clear interpretation of the data, the value of the free fatty acids was subtracted from the total lipids. This gave a value corresponding to the total weight of simple lipids minus free fatty acids, termed remaining simple lipid. From Table 24 it may be observed that the remaining simple lipid decreased from 285 to 248 mg/100 g powder for 7°C storage, while for 30°C storage this decrease was from 285 to 203 mg/100 g powder (Table 25). Both losses were found to be significant, although the 30°C storage produced larger losses. There was a certain amount of uniformity in the decrease pattern of the remaining simple lipids in cans stored at 7°C, whereas this decrease was somewhat erratic in cans stored at 30°C.

Monoglycerides (MG) and diglycerides (DG) showed the least retention when viewed on a percentage retention basis when stored at both 7 and 30°C (Fig. 40). Values of 50% and 30% were calculated for the retention of MG at 7 and 30°C storage temperatures respectively after 12 months. Seventyfive percent and 55% retention values were found in DG. Triglycerides (TG), FS, and ES all showed

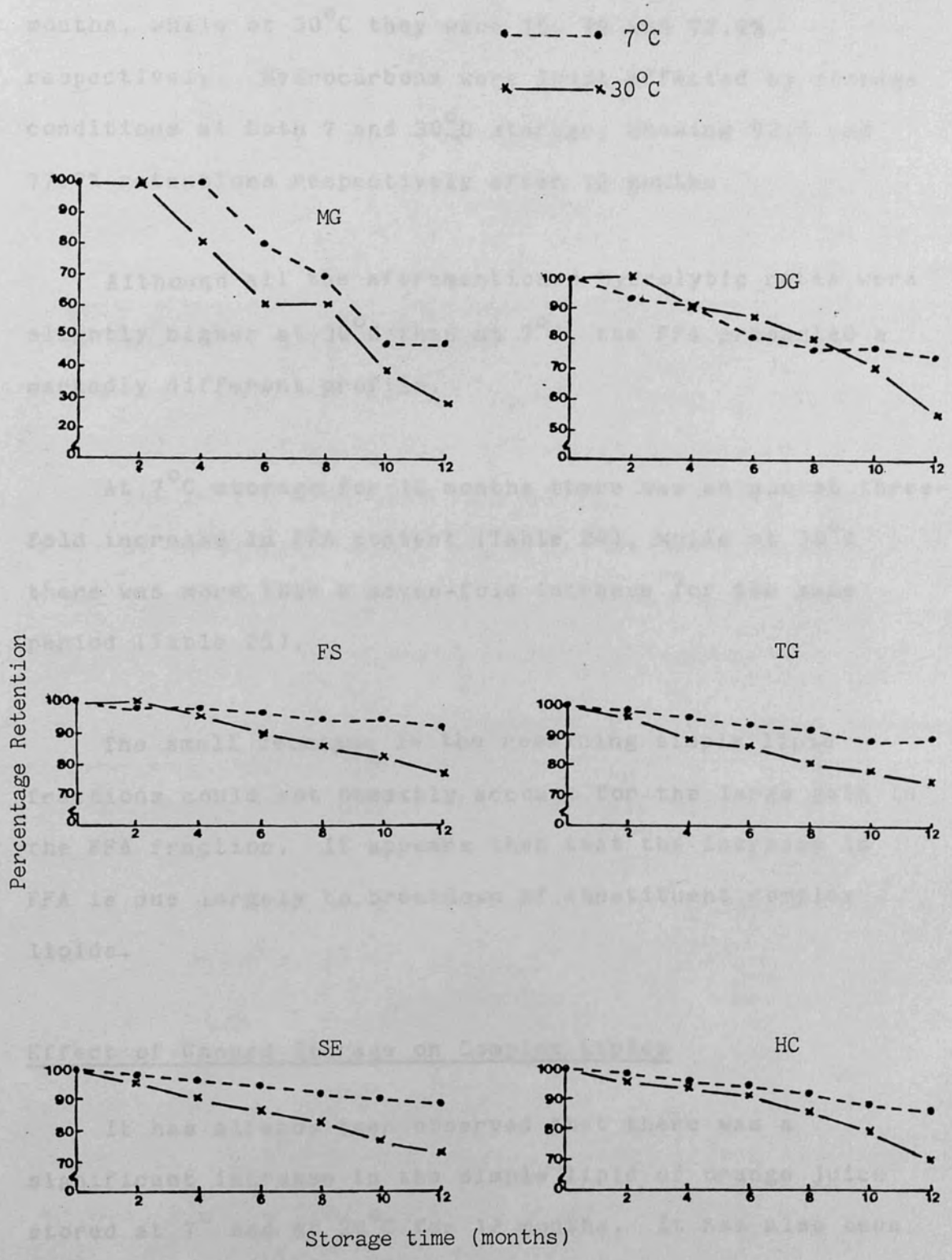


Fig. 40. Percentage retention of simple lipids of canned Sia-Varaz orange juice stored at 7° and 30°C over a twelve months period. For signficant differences refer to Tables 24 & 25

retentions of approximately 88% at 7°C storage after 12 months, while at 30°C they were 75, 70 and 72.7% respectively. Hydrocarbons were least affected by storage conditions at both 7 and 30°C storage, showing 92.4 and 77.2% retentions respectively after 12 months.

Although all the aforementioned hydrolytic rates were slightly higher at 30°C than at 7°C, the FFA presented a markedly different profile.

At 7°C storage for 12 months there was an almost three-fold increase in FFA content (Table 24), while at 30°C there was more than a seven-fold increase for the same period (Table 25).

The small decrease in the remaining simple lipid fractions could not possibly account for the large gain in the FFA fraction. It appears then that the increase in FFA is due largely to breakdown of constituent complex lipids.

Effect of Canned Storage on Complex Lipids

It has already been observed that there was a significant increase in the simple lipid of orange juice stored at 7° and at 30°C for 12 months. It has also been shown that this substantial increase was the result of a significant increase in the free fatty acids which may have come from certain complex lipids. The effect of

storage of SSOJ at 7° and at 30°C for 12 months will now be discussed.

Total complex lipids of canned orange juice was 980 mg/100 g powder at first. This decreased to 843 mg/100 g powder after 12 months' storage at 7°C (Table 26) indicating a significant loss of 137 mg/100 g powder. This loss was fairly uniformly distributed throughout the individual component complex lipids in terms of percentage loss (Fig. 41), ranging from 75% retention of PI to 90% retention of PA. The only complex lipid component not affected by storage at 7°C was LPC. Breakdown of the other lipids would contribute in a further build up in the pool of free fatty acids already mentioned.

Among the individual free fatty acids C16:0 appeared to have increased the most from 10 to 30 mg/100 g powder, whereas C18:3 has increased the least from 5 to 13 mg/100 g powder after 12 months of storage at 7°C (Table 27 and Fig. 42).

The higher temperature of storage (30°C) has a marked effect on the lipids in canned orange juice. Also some deterioration of the lacquer coating within the cans was noticed from four months of storage onwards.

It can be seen from Table 28 that on an absolute weight basis PC and PE showed the largest losses;

Table 26. Effect of storage at 7°C over a twelve months period on the complex lipids of canned single-strength orange juice. Graphical presentation of the data is shown in Fig. 41

Sampling time (months)	Complex lipid content (mg/100 g powder)										Total complex lipid (mg/100 g powder	Complex lipid % of total	Phospho-lipids % of complex	Glyco lipids % of complex
	PI	UNK	LPC	PC	PS	PG	PE	PA	MGDG	CMH				
0	45.9 efg	13.3 efg	25.2 n.s.	245.9 bcdefg	37.1 bcdefg	20.7 defg	288.3 bcdefg	63.7 bcdefg	142.7 n.s.	96.8 n.s.	980.1 cdefg	73.3	75.6	24.4
2	43.0 cdef	13.0 ef	25.8 n.s.	239.7 abcdef	36.7 abcdef	19.8 def	280.5 abcdef	63.3 bcdef	142.1 n.s.	96.0 n.s.	959.9 cdef	74.8	75.2	24.8
4	40.3 bcde	12.2 cde	26.2 n.s.	234.3 abcde	36.0 abcde	18.8 abcd	273.8 abcde	61.6 abcde	140.2 n.s.	95.0 n.s.	938.7 abcde	74.2	74.9	25.0
6	39.0 abcd	11.0 abcd	26.0 n.s.	228.1 abcd	37.0 abcd	19.1 abcde	269.0 abcd	60.5 abcd	140.2 n.s.	93.1 n.s.	922.5 abcd	73.5	74.8	25.3
8	37.0 abc	10.5 abc	25.1 n.s.	221.4 abc	35.1 abc	16.3 abc	260.1 abc	58.5 abc	135.2 n.s.	92.6 n.s.	891.8 abc	72.4	74.4	25.5
10	35.4 ab	9.4 ab	27.3 n.s.	212.7 ab	33.9 ab	14.7 ab	253.3 ab	57.0 ab	131.7 n.s.	92.0 n.s.	867.4 ab	71.6	74.2	25.8
12	34.3 a	9.3 a	27.5 n.s.	205.0 a	33.0 a	12.1 a	248.5 a	55.3 a	124.3 n.s.	90.5 n.s.	843.4 a	71.0	74.1	25.5

n.s. = not significant

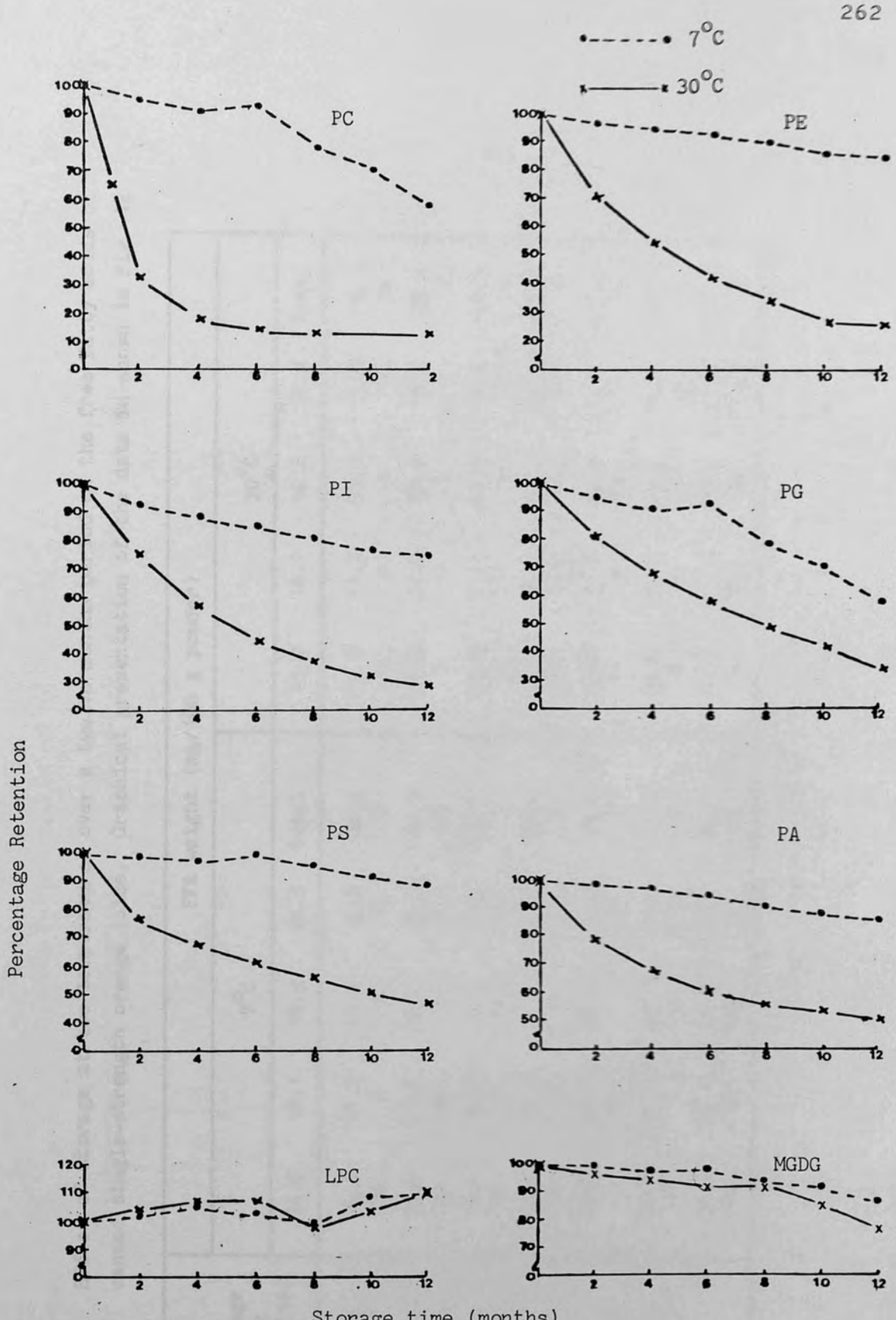


Fig. 41. Percentage retention of phospho- and glycolipids of canned Sia-Varaz orange juice stored at 7° and 30°C over a twelve months period. For significant differences refer to Tables 26 and 28

Table 27. Effect of storage at two temperatures over a twelve months period on the free fatty acids of canned single-strength orange juice. Graphical presentation of the data is shown in Fig. 42

Storage time (months)	FFA weight (mg/100 g powder)									
	7°C					30°C				
	16.0	18.1	18.2	18.3	Total	16.0	18.1	18.2	18.3	Total
0	10.0 a	11.2 a	10.0 a	5.3 a	36.5 a	10.0 a	11.2 a	10.0 a	5.3 a	36.5 a
2	12.6 ab	13.2 ab	10.8 ab	6.0 ab	42.6 ab	28.3 b	25.0 b	29.0 b	16.5 b	98.8 b
4	15.4 bc	15.7 bc	13.6 bc	7.9 bc	52.3 c	48.0 c	41.6 c	44.8 c	30.5 c	166.5 c
6	20.6 d	18.1 cd	18.1 d	10.6 d	67.4 d	54.1 cd	46.3 cd	56.8 d	33.3 cd	190.5 d
8	27.2 e	20.2 de	22.0 e	12.0 dd	81.4 e	60.3 de	55.0 de	63.6 de	41.1 e	220.0 e
10	30.1 ef	24.7 f	24.0 ef	14.0 efg	92.8 f	66.8 ef	57.9 ef	69.2 f	46.3 ef	240.2 f
12	30.0 efg	28.0 fg	24.8 efg	13.4 ef	96.1 fg	74.9 fg	63.3 efg	77.6 fg	52.6 g	268.4 g

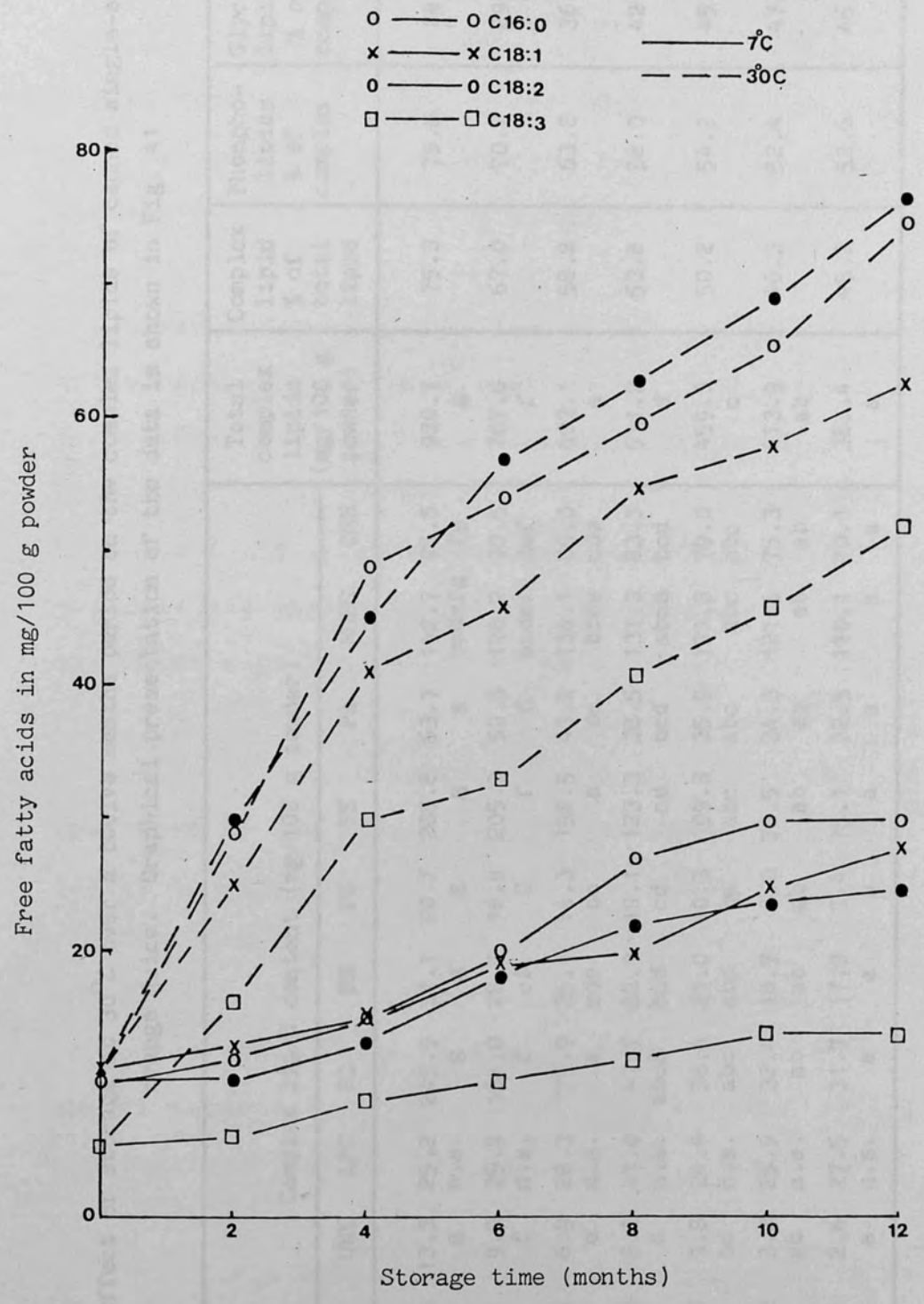


Fig. 42. The effect of storage at two temperatures over a twelve months period on free fatty acids of canned single-strength orange juice. For significant differences refer to Table 27

Table 28. Effect of storage at 30°C over a twelve months period on the complex lipids of canned single-strength orange juice. Graphical presentation of the data is shown in Fig. 41

Sampling time (months)	Complex lipid content (mg/100 g powder)										Total complex lipids (mg/100 g powder)	Complex lipid % of total lipid	Phospho-lipids % of complex	Glyco-lipids % of complex
	PI	UNK	LPC	PC	PS	PG	PE	PA	MGDG	CMH				
0	45.9 g	13.3 g	25.2 n.s.	245.9 g	37.1 g	20.7 g	288.8 g	63.7 g	142.7 bcdefg	96.8 fg	980.1 g	75.3	75.6	24.4
2	34.1 f	9.9 f	25.9 n.s.	167.0 f	28.7 ef	16.9 f	205.9 f	50.5 f	128.2 bcdef	90.5 def	767.6 f	67.0	70.2	29.8
4	26.5 e	6.9 e	26.3 n.s.	79.9 e	25.4 cde	14.3 de	158.5 e	43.2 de	136.1 bcde	86.0 cde	603.1 e	58.8	63.2	36.8
6	20.3 cd	5.1 d	27.0 n.s.	47.7 abcd	22.9 bcd	12.1 cd	123.3 cd	38.5 bcd	131.3 abcd	83.3 bcd	511.2 d	53.8	58.0	42.0
8	17.4 abc	3.8 bc	24.9 n.s.	36.4 abc	21.0 abc	10.3 bc	99.3 abc	35.9 abc	131.3 abc	79.0 abc	459.3 c	50.2	54.2	45.8
10	15.2 ab	3.0 ab	25.9 n.s.	32.4 ab	18.7 ab	9.0 ab	78.5 ab	34.3 ab	121.6 ab	75.3 ab	413.9 ab	46.3	52.4	47.6
12	14.1 a	2.6 a	27.5 n.s.	31.7 a	17.3 a	7.4 a	75.1 a	32.5 a	110.1 a	70.1 a	388.4 a	45.2	53.6	46.4

n.s. = not significant

214 and 214 mg/100 g powder in both cases after 12 months' storage. Retention of all the phospholipids was low after 12 months of storage, being 30% for PI, 13% for PC, 47% for PS, 36% for PG, 26% for PE and 51% for PA. DGDG has been the most affected among the glycolipids, showing only 40% retention (Fig. 41).

Lysophosphatidyl choline showed a slight increase which reflected the breakdown of PC. The absence of lyso-derivatives for the other phospholipids implied that breakdown of the latter was complete and suggested further that the fatty acids associated with the phospholipid molecule had been split off. The presence of LPC indicates a gradual degradation of PC.

Such a large breakdown of phospholipids was also indicated by the change in the percentage of complex lipids to total lipids from 75.3 to 45.2% (Table 28).

Changes in the phospholipid fractions were more pronounced than those in simple lipids implying that steryl esterases and neutral lipases were not as active as phospholipases which were very active indeed.

The largest increase in free fatty acids, in canned juice stored at 30°C, occurred between 0 - 2 months and 2.4 months of storage. They were 62 and 68 mg/100 g powder respectively (Table 27 and Fig. 42). This was

related to the hydrolysis of PC and PE which was highest within the first four months of storage. There was, however, very little variation in the unsaturation ratio recorded for fatty acids throughout the storage period indicating that the ratio of C16:0 to the unsaturated fatty acids remained fairly constant.

The fact that off-flavour had developed and that discolouration of the can's interior had occurred, coupled with a substantial build up of free fatty acids, caused by the breakdown of phospholipids, provided evidence that some of the fatty acids may have undergone various degradative reactions to form odorous compounds. Hence it is likely that lipid breakdown plays a vital role in producing the off-flavours in canned orange juice stored at 30^{o}C. The exposed metal of the can's interior would also provide a means by which trace elements could be in contact with the juice, thereby enhancing lipid oxidative reactions, hence causing further deterioration of the juice.

CHAPTER XII

General Conclusions on the Canning and Storage of Orange Juice

Free Sugars

No significant change in total free sugars was observed between fresh and canned Sia-Varaz orange juice. Four individual sugar components were identified in the total sugar fraction, namely glucose, fructose, galactose and sucrose. Colour of the juice was not impaired after processing.

Canned storage for twelve months at 7°C did not lead to any significant loss in total sugar content. However, changes in individual sugars were noted, in that while galactose remained constant, sucrose decreased by 40%, producing increases in glucose and fructose of 22 and 29% respectively. This sucrose hydrolysis was initiated by acidic conditions prevalent in the juice.

Canned storage at 30°C led to significant losses in total free sugars, giving only 72% retention after twelve months. Sucrose was the most affected in that it was completely absent after ten months. Glucose did not show an overall increase after twelve months while fructose increased by 1.9 g/100 ml.

The 3.0 g/100 ml loss in free sugars may have been due to further degradation reactions of the reducing sugar components. Also these reactions appeared to preferentially affect glucose and not fructose. Evidence was offered that some of the glucose had taken part in Maillard reaction. Furthermore, there was a substantial five-fold increase in optical density. There was a lag period of one month followed by an increase at an almost constant rate leading to severe discolouration of the juice.

A combination of dehydration of the sugars to form HMF and direct involvement of reducing sugars with free amino acids in the Maillard reaction appeared to have been the prime cause for loss in glucose and fructose. This led to the formation of stable pigmented compounds which on accumulation accounted for the observed darkening of the juice. HMF levels were found to increase markedly in the aged juice.

Vitamin C

No significant effect on vitamin C content was noted as a result of canning the orange juice.

Storing canned juice at 7°C for twelve months has resulted in no significant loss of vitamin C, nor any deterioration in the flavour and colour. One glass (177 ml) of this juice after twelve months was still

sufficient for the RDA of 60 mg. Low temperature storage was therefore beneficial in maintaining nutrients.

Stored cans of juice for twelve months at 30°C showed significant losses of vitamin C. Significant losses were noticed within the second week of storage. This rapid loss was caused by the presence of free residual oxygen remaining in the headspace of the can and soluble oxygen within the juice. After this initial period the rate of loss of vitamin C appeared to be slower due to the fact that aerobic degradation occurs at a more rapid rate than anaerobic degradation.

This vitamin C breakdown, and a concomitant build-up of furfural and its subsequent reaction with free amino acids may have contributed to the darkening of the juice. Fructose and trace amounts of metals from the can may play a part in enhancing the rate of breakdown of vitamin C.

One glass of the juice stored at 30°C for more than four months was not sufficient for the RDA. Flavour is also dramatically impaired after this time. In general, it can be said that at 30°C both breakdown of vitamin C and the reaction of free sugars with free amino acids are the main contributors to the darkening of the juice.

Free Amino Acids

Seventeen free amino acids were identified in fresh Sia-Varaz orange juice. Canning brought about a 193.6% increase in total free amino acids. This increase was attributed to the breakdown of lipo-proteins in the "cloud", probably caused by the elevated temperatures during pasteurization. There was no qualitative change in free amino acids. On a quantitative basis, histidine and methionine were observed to decrease, tyrosine remained constant while all remaining amino acids increased. Cysteine, which was found as a trace in the fresh juice, appeared in slightly larger amounts in the processed juice.

Storing canned juice at 7°C did not bring about any significant change in total free amino acids, nor in the individual amino acids of processed juice. This suggests that the canning of juice and subsequent storing at 7°C gives an overall beneficial effect to the amino acid status of the orange juice.

Storing canned juice at 30°C resulted in a significant loss in total amino acid content of 19%. Although there were no qualitative changes in individual amino acids, their relative amounts fluctuated appreciably.

There was relatively high levels of free sugars and vitamin C in freshly canned orange juice, which then decreased substantially after storage for twelve months

at 30°C. It was suggested that a certain amount of this loss could have accounted for the browning reactions. This, with the observation that appreciable amounts of amino acids were also lost, implied that the sugars and vitamin C may have reacted with some of the free amino acids in the Maillard reaction to form brown pigments.

Lipids

Canning Sia-Varaz orange juice brought about no significant increase in simple lipids. Individual simple lipid components also did not increase. Total complex lipids increased significantly of which glycolipids were unaffected. It was mostly the phospholipids, phosphatidyl choline and phosphatidyl ethanolamine which increased.

The likely explanation for the observed increase in phospholipids was that the treatment during pasteurization may have caused disruption of the membrane structure in the "cloud", thereby facilitating lipid extraction.

Storing canned orange juice at 7°C brought about a significant loss in total lipids after eight months but no change in flavour of the juice was noted even after twelve months of storage. Simple lipids showed a significant decrease after six months of storage but with a three-fold rise in free fatty acid content.

There was also a significant loss in complex lipids after six months.

It appears that, although free fatty acids were being released due to hydrolysis of other lipid components, these high carbon number fatty acids contributed very little to flavour. Their importance is as precursors to many volatile off-flavour compounds, hence this gave the impression that little fatty acid degradation was taking place.

Storing canned juice at 30°C brought about a significant loss in total lipids within the second month. The overall decrease was most rapid in the first four months. There was a significant increase in simple lipids in that before storage, they represented 24.7% of total lipids but after storage for twelve months, they represented 54.8%. This increase was solely due to a seven-fold rise in free fatty acid content. Flavour deterioration was also noted after this time.

The general conclusion then is that canned Sia-Varaz single-strength orange juice may be kept for twelve months at 7°C without being obviously affected. However, if kept for more than four months at 30°C the colour, flavour and vitamin C content have been affected so much that the juice cannot be consumed. Therefore, the inhabitants of Southern Iran would do well not to keep canned orange juice too long unless it is kept in a refrigerator.

ACKNOWLEDGEMENTS

I would like to express my grateful thanks to my Supervisor, Dr L R G Valadon, for his invaluable and unstinted help, his keen interest and patience throughout this study.

My thanks also go to all members of the academic and technical staff of the Botany Department and my post-graduate student friends for their willingness to help.

My thanks are due to my parents for their constant support and encouragement.

My thanks also go to Mr Younes Ebrahimi, Eng., Head of Rumsar Experimental Station (Iran) for his regular supply of the samples without which this study would not have been completed.

My thanks also go to Mrs Inge Judd for her excellent care in typing the manuscript.

Finally, to my wife Pari-sima I would like to say "thank you for making it possible".

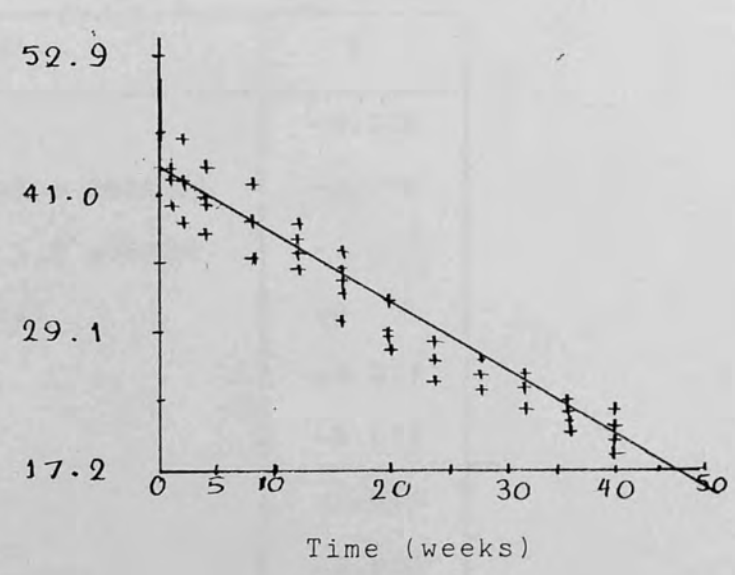
A plot of vitamin C concentration against time showed a curvilinear regression. A least square estimate for a straight line relationship (ANOVA 1; graph 1) shows the obvious curvilinear relationship.

AOV: LINEAR REG: COD 1

SOURCE/DF	SS	MS	F
TOTAL 59	5445.4		
REG 1	5020.1	5020.1	684.7
RESID 53	425.3	7.3	

R SQUARE = 0.922

YHAT = 43.134 − 0.575 X



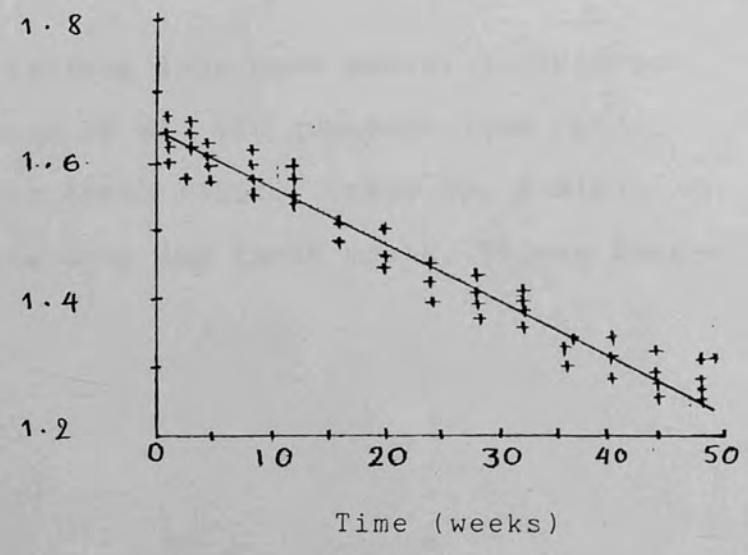
A plot of log vitamin C content against time showed a better fit to a linear regression with an improvement in the reduction of the error mean square (ANOVA 2, graph 2).

AOV: LINEAR REG: CODE 1

SOURCE/DF	SS	MS	F
TOTAL 59	1.1		
REG 1	1.1	1.1	999.9
RESID 58	0.1	0.0	

R SQUARE = 0.955

YHAT = 1.643 − 0.008X



By taking results in sets of 3 and calculating the slope, a table of b values for each triplet of results shows that the value decreased from -0.025 in month 1 to -0.005 in month 12.

Table 17a - The b values

Time	b
0,1,2 weeks	-0.025
1,2 weeks, and 1 month	-0.010
2 weeks, and 1,2 months	-0.006
1,2,3 months	-0.005
2,3,4 "	-0.011
3,4,5 "	-0.012
4,5,6 "	-0.010
5,6,7 "	-0.008
6,7,8 "	-0.005
7,8,9 "	-0.010
8,9,10 "	-0.009
9,10,11 "	-0.005
10,11,12 "	-0.005

Calculating the monthly loss rate showed a variation from 4 - 15% with a mean of 8%, the greatest loss rate occurred over the first month (15%). (Table 17, p.218). Analysing the loss rate over the first month, 9% was lost in the first week.

However the straight-line relationship of graph 2 is not perfect and suggests that the loss rate in the first month is higher than the general slope (Table 17b) and therefore this gives a steeper slope for the first month of the storage at 30°C.

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