450.5 m μ .) since it yielded a single band on mixed chromatography with cryptoxanthin obtained from yellow maize (Sadana, 1946).

Zone II. This was an orange-coloured band. The pigment was identified as neo- β -carotene U (maxima in light petroleum 480 and 450 m μ .; Sadana, 1949).

Zone III. This was reddish orange in colour. The pigment was extracted and identified as β -carotene by its absorption spectrum (maxima in light petroleum 482 and 452 m μ .).

Zone IV. This was a brownish yellow band. The pigment was extracted and identified as neo- β -carotene B (maxima in petroleum 470 and 443 m μ .). It yielded a single band on mixed chromatography with neo- β -carotene B prepared by HCl treatment of β -carotene (Sadana, 1949).

Results of analyses of six varieties of loquats varying in colour from pale yellow to deep orange are shown in Table 1.

SUMMARY

Pigments separated chromatographically and identified from different varieties of loquats include, besides 'xanthophylls other than cryptoxanthin', cryptoxanthin, β -carotene, neo- β -carotene U and neo- β -carotene B. Among the pigments present, cryptoxanthin and β -carotene are the principal ones forming 15.7–33.8 and 36.5–59.6% of the total pigments.

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Improved Separation of Sugars on the Paper Partition Chromatogram

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Our object has been to develop methods for the quantitative analysis of the mixtures of sugars present in the polysaccharides of the cell wall of fruits; these mixtures frequently include glucose, galactose, xylose, mannose and arabinose, as well as galacturonic and glucuronic acids. The work of Partridge (1946, 1948) has demonstrated the effectiveness of the paper chromatogram as a means of separating reducing sugars, whilst the work of Hirst, Jones & Flood (1947) and Hawthorne (1947) has shown that the amounts of sugars can be estimated within $\pm 5\%$ once they have been separated on the paper chromatogram.

It was found in preliminary experiments that none of the solvents previously used in this work was in fact capable of separating all the sugars known to be present in the mixtures under investigation. A large number of solvents was then investigated, and this study led not merely to the development of two new solvents having the required specificity, but also to a more selective method of operating the paper partition chromatogram. The new method involves the use of solvents in which sugars give R_f values of about 0.2. Under such conditions the selectivity defined in terms of the ratio of the R_f values for the pair of sugars to be separated is much improved. Separation of sugars with such low R_f values would need exceedingly long papers if run in the normal manner, but by allowing the solvent to drip off the end of the paper

the difficulty has been avoided. Solvents are chosen which have a low viscosity so that the solvent flows quickly through the paper.

To obtain solvents which give R_f values about 0.2 for any particular pair of sugars, it is convenient to use three-component systems so that the partition coefficient, and with it the R_f value, can be adjusted to the desired value. Such solvents have been used previously by Martin & Synge (1941), Isherwood (1946) and Partridge (1946, 1948). Usually two of the components are immiscible whereas the third is miscible with either of the other two. By varying the amount of the third component it is possible to adjust the partition coefficient over a wide range. The two new solvents, ethyl acetate-pyridine-water and ethyl acetate-acetic acid-water are mixtures of three components and in addition have a low viscosity.

EXPERIMENTAL

Apparatus. The apparatus was similar to that described by Partridge (1948). The main difference was that a small hole was bored through the lid of the chamber immediately above one end of the stainless steel trough so that a pipette could be inserted to deliver solvent to the trough. The hole was normally closed with a tight fitting bung. Whatman no. 1 filter paper was used for the strip chromatograms, one end being cut to a point to facilitate even drainage when the solvent front had reached the bottom and was dripping from the paper. The sugar solutions used were roughly 1% (w/v) with respect to each individual sugar and were

applied to the paper with a micropipette (3–7 μ l.) so that the size of the spots could be standardized. The whole apparatus was kept in a constant-temperature room at $20^\circ \pm 0.25^\circ$, as it was found that changes in temperature altered the R_F values for the sugars in an irregular manner, especially if the critical temperature of the mixture of solvent and water was close to that of the room.

Reagents. The phenol and *s*-collidine were purified as described by Partridge (1946, 1948). The *sec*.-butanol was purified by distillation through a vacuum-jacketed fractionating column, the middle third of the distillate being used for the paper chromatograms. This fraction had a constant boiling point. *iso*Butyric acid was treated similarly. The critical temperature of a mixture of the purified *isobutyric* acid and water was found to be 24.3° . The accepted figure is 24.5° .

Solvents which contained only one component besides water were equilibrated against water at 20° . In the case of solvents containing three components, definite volumes of each component were mixed and when equilibrium between the two phases that resulted had been attained, the non-aqueous phase was separated and used as solvent without further treatment.

Procedure. The spots of sugar solution were added to the paper from a micropipette (3–7 μ l.) in a similar manner to that described by Hawthorne (1947). The papers were allowed to dry, and were then hung from the trough in the chamber, no solvent being present. Sufficient of the non-aqueous and aqueous phases was now added to the bottom of the chamber to ensure that the papers would reach equilibrium with the solvent. The lid was placed in position and the apparatus left overnight. After this preliminary equilibration the bung was removed from the small hole in the lid, and solvent added to the trough from a pipette, care being taken to avoid loss of the vapour from inside the chamber. The bung was then replaced. The subsequent treatment of the papers follows closely that described by Partridge (1948).

RESULTS

Variation of R_F value with solvent

Results for rhamnose, xylose and glucose are recorded in Table 1. These sugars were chosen because they were conveniently spaced within the range of R_F values obtained for the simple sugars. The solvent front was not allowed to run off the paper.

These results showed that the absolute R_F values were directly dependent upon the molar fraction of water in the non-aqueous phase. They also showed that, as the molar fraction of water in the non-aqueous phase decreased, the ratio of the R_F values for the pairs of sugars approached a definite maximum. For practical purposes this maximum was reached when the R_F values were less than 0.2, which meant that the possibility of separating two similar sugars increased as the absolute R_F values were reduced to 0.2. A paper chromatogram operated under this restriction did not give a sufficient separation of the sugars if the solvent front remained on the paper because the distance between the centres of the spots was often not more than 1–2 cm. However, if the solvent front was allowed to run off the paper so that the spots moved 10–20 cm. then the distance between the centres increased to 5–10 cm.

Separation of the sugars

From the preceding paragraph it appears that the best conditions for the separation of the sugars are when the R_F values are less than 0.2 and the solvent front is allowed to run off the end of the paper so that the spots move 10–20 cm. The main drawback is that the time required for the spots to move apart on the paper can be considerable if the solvents used have a high viscosity, e.g. *cyclohexanol*. It is essential, therefore, to use solvents which have a low viscosity in order that the solvent front may move as quickly as possible. A comparison of the viscosity of various solvents with the rate of movement of the solvent front is given in Table 2. The viscosities given are those for pure liquids while the velocity of the solvent front is for the same liquid saturated with water. The influence of viscosity on the separation of the sugars has been calculated by combining the figures for the velocity of the solvent front with the R_F values given in Table 1, the assumption being made that the rate of flow of solvent through the paper and the R_F value remain constant.

Table 1. Variation of R_F values of sugar with solvent at 20°

Solvent	Water in non-aqueous phase. Mol. fraction	R_F value			R_F Rhamnose R_F Xylose	R_F Xylose R_F Glucose
		Rhamnose	Xylose	Glucose		
Ethyl acetate	0.132	0.01	0.00	0.00	—	—
Methyl acetate	0.269	0.075	0.04	0.00	1.9	—
Methyl ethyl ketone	0.305	0.095	0.05	0.0025	1.9	2.0
Benzyl alcohol	0.387	0.11	0.06	0.03	1.8	2.0
<i>cyclo</i> Hexanol	0.432	0.15	0.06	0.03	2.5	2.0
<i>m</i> -Cresol	0.471	0.27	0.15	0.07	1.75	2.1
<i>n</i> -Butanol	0.508	0.22	0.125	0.07	1.7	1.8
<i>tert</i> .-Amyl alcohol	0.609	0.32	0.245	0.145	1.30	1.7
Phenol	0.668	0.59	0.44	0.39	1.34	1.13
Quinoline	0.680	0.41	0.31	0.19	1.32	1.6
<i>sec</i> .-Butanol	0.707	0.41	0.31	0.26	1.32	1.27
<i>iso</i> Butyric acid	0.778	0.51	0.44	0.37	1.16	1.19
<i>s</i> -Collidine	0.846	0.59	0.50	0.44	1.18	1.14

Table 2. Calculated distance the centre of a sugar spot should move in 24 hr. for different solvents at 20°

Solvent	η (Centipoises)	Velocity of solvent front (cm./hr.)	Distance (cm.)			
			Solvent front	Rhamnose	Xylose	Glucose
Methyl acetate	0.38	12	288	21.6	11.5	0.0
Ethyl acetate	0.45	10	240	2.4	0.0	0.0
Methyl ethyl ketone	0.43	10.8	258	25.5	12.9	6.4
Water	1.0	3.75	—	—	—	—
isoButyric acid	1.3	2.25	54	27.5	23.7	19.9
n-Butanol	3.0	2.6	62	13.6	7.8	4.4
sec.-Butanol	3.7	2.6	62	25.6	19.2	16.2
tert.-Amyl alcohol	4.7	1.25	30	9.6	7.3	4.3
Benzyl alcohol	5.8	1.6	38.4	4.2	2.3	1.1
Phenol	11.7	1.5	36	21.2	15.8	14.1
m-Cresol	20.0	1.1	26.5	7.1	4.0	1.8
cycloHexanol	68.0	0.65	15.6	2.3	0.9	0.4

Table 3. Separation of sugars using three-component solvents

Mixture prepared (in vols.) Non-aqueous phase used as solvent	Duration of test (hr.)	Movement of the centre of the sugar spot from the starting point (cm.)							
		Rhamnose	Xylose	Arabinose	Mannose	Fructose	Glucose	Galactose	Galact- uronic acid
tert.-Amyl alcohol 3 Water 3 Acetic acid 1	20	8.6	7.0	5.4	5.9	5.9	4.6	4.1	3.2
Benzyl alcohol 3 Water 3 Acetic acid 1	22	12.8	7.8	7.5	5.7	7.2	4.7	4.6	2.6
Chloroform 4 *Water 4 Methanol 7	24	28.6	23.3	20.5	18.6	19.3	16.3	15.9	6.6
Ethyl acetate 16 *Water 16 Methanol 9	14	6.5	5.4	4.7	3.8	4.0	3.2	3.1	1.1
Ethyl acetate 2 Water 2 Pyridine 1	22†	37.9 (0.49)	33.6 (0.38)	29.2 (0.33)	28.9 (0.32)	28.4 (0.32)	26.0 (0.28)	20.9 (0.235)	6.7 (0.025)
Ethyl acetate 3 Water 3 Acetic acid 1	39†	28 (0.34)	25.3 (0.265)	20.5 (0.22)	17.6 (0.195)	—	14.3 (0.17)	12.4 (0.14)	12.0 (0.13)
Ethyl acetate 2 *Water 2 Dioxan 3	14	30.1	26.2	22.4	22.4	21.3	20.8	17.7	5.1

* These mixtures only just formed two phases, and as the presence of the sugar markedly increased the mutual solubility of the two phases, the spots 'tailed' badly. The separation between the sugar spots was, therefore, less than the figures suggest.

† R_F values are given in parentheses.

In practice the separations achieved are much less than is shown in Table 2, the main reason being that when the solvent commences to drip from the end of the paper, the rate of flow of solvent is reduced to about half the initial value. The relative separations, however, remain substantially the same, though they do not entirely correspond to the R_F values measured when the solvent front is still on the paper. The explanation probably lies in the fact that the distribution of the solvent throughout the paper strip is modified if liquid accumulates at the bottom (cf. Consden, Gordon & Martin, 1944).

None of the solvents listed in Table 2 is ideal. One

or two give excellent separations of the pentoses, but do not give an appreciable separation of the hexoses in a convenient time (24 hr.). The addition of a third component which is soluble in both phases will tend to increase the solubility of hexoses in the non-aqueous solvent and thereby increase their R_F values. The separation of the pentoses will be relatively less affected. The results obtained with a number of three-component mixtures are given in Table 3.

Of these mixtures ethyl acetate-pyridine-water and ethyl acetate-acetic acid-water were selected as they fulfilled the requirements for which this work was undertaken.

Analysis of mixtures of sugars

In a mixture containing arabinose, xylose, glucose, galactose, galacturonic acid and mannose, it was possible to detect all the components with certainty using one or other of the above solvent mixtures. Of these sugars arabinose could not be distinguished from mannose in ethyl acetate-

or by the method described by Hawthorne (1947). We preferred, however, a combination of the two methods. A known amount of the sugar solution was added to the paper from a micropipette (Hawthorne, 1947), and then the sugars separated and isolated following the method described by Hirst *et al.* (1947). Analysis of four mixtures by this method is given in Table 4.

Table 4. *Analysis of mixtures of sugars*

	Amount added (μ g.)	Amount recovered (μ g.)				
		Individual experiments				Mean
Mixture 1						
Xylose	68.5	61.6	70.4	74.0	67.2	67.0
Arabinose	68.5	77.4	71.3	67.8	67.9	71.1
Mannose	68.5	69.4	76.4	67.3	65.5	69.4
Glucose	68.5	67.9	59.1	77.6	65.3	67.5
Galactose	68.5	74.4	68.9	66.6	71.8	70.4
Mixture 2						
Xylose	68.5	71.0	66.4	75.0	68.1	70.1
Arabinose	68.5	68.0	69.4	74.1	70.4	70.4
Glucose	68.5	75.4	71.1	66.8	64.1	69.3
Galactose	68.5	74.6	61.9	77.1	65.2	69.8
Galacturonic acid	68.5	63.2	85.8	74.2	73.6	74.2
Mixture 3						
Xylose	212	222	215	217	211	216
Arabinose	24.0	25.5	29.2	30.1	20.1	26.4
Glucose	439	433	428	421	426	427
Galacturonic acid	44.3	39.4	46.1	41.8	44.7	43.0
Mixture 4						
Xylose	19.0	19.8	18.4	21.0	23.4	20.7
Arabinose	414	415	391	421	400	406
Glucose	39.8	33.9	45.3	42.1	38.4	39.9
Galacturonic acid	204	208	200	210	214	208

pyridine-water, and galactose and galacturonic acid could not be separated in ethyl acetate-acetic acid-water. If a pair of sugars had very different R_f values separation was completed very rapidly. Thus with ethyl acetate-pyridine-water a mixture of glucose and xylose was completely separated in 2 hr. If only a small proportion of one sugar was present it was advantageous to work with a concentrated solution though the more concentrated the solution used the larger the spot after development. The use of concentrated solutions was possible with the solvents described above because the distance between the centres of the sugar spots was 3–5 cm. Thus, using ethyl acetate-acetic acid-water as solvent, mannose was identified in the presence of 10 times its concentration of glucose and 100 times its concentration of xylose. The total concentration of sugars in the solution used for making the spot was 20% (w/v), and the diameter of the xylose spot after the chromatogram was developed was 4–5 cm.

Quantitative analysis of the sugars on the paper chromatogram after they had been separated using either of these solvents was carried out successfully either by the method described by Hirst *et al.* (1947)

Mixture 1 was separated using ethyl acetate-acetic acid-water as solvent. The time required for the separation of the sugars was 48 hr. Mixtures 2, 3, 4 were separated using ethyl acetate-pyridine-water as solvent. In the case of mixtures 3 and 4 the composition of the solvent was slightly modified, the components being in the ratio of 5, 2, 5 instead of the ratio of 2, 1, 2 respectively as described in Table 3. The modified solvent gave slightly better separations than the original. The papers were dried at room temperature overnight because it was found that drying at the usual temperature of 60–100° in the presence of pyridine appeared to destroy a large part of the sugar present.

Mixtures 3 and 4 are examples of the separations achieved when some of the sugars are present only in small proportions.

DISCUSSION

If we consider the separation of the sugars after they have moved a definite distance down the paper chromatogram we find that the best separations are obtained with solvents which give R_f values less

than 0.2, the solvent being allowed to run off the end of the paper. This observation is in agreement with predictions which can be made by applying to the paper chromatogram the equation first deduced by Martin & Synge (1941) for the relation between the complete separation of two solutes (99.8% of one and 0.2% of the other) and the characteristics of the partition column:

$$\frac{A_L + \alpha A_s}{A_L + \beta A_s} = \frac{r - 3\sqrt{r+2.25}}{r + 3\sqrt{r+2.25}},$$

where α and β are the partition coefficients of the two solutes, A_L is the area of cross-section of the mobile phase, A_s is the area of cross-section of the non-mobile phase and r represents the number of 'theoretical plates' the first solute has passed. At

any particular place in the column $\frac{r - 3\sqrt{r+2.25}}{r + 3\sqrt{r+2.25}}$ is

a constant and represents the maximum value that $(A_L + \alpha A_s)/(A_L + \beta A_s)$ can have if the two solutes are to be just separated. If A_L is small in relation to αA_s or βA_s the expression reduces to α/β . This is true when the partition coefficients are large. Now if we assume that the ratio α/β is likely to be sensibly constant for solvents of a similar type it follows that $(A_L + \alpha A_s)/(A_L + \beta A_s)$ reaches its minimum value when the partition coefficients are large, and that if two solutes are only just separated when the partition coefficients are large they will not be separated when the partition coefficients are small. The assumption that the ratio α/β is sensibly constant for solvents of a similar type is strongly suggested by analogy with the data given by Cohn & Edsall (1943) on the solubility of amino-acids and their derivatives in various organic solvents and in water. It is important, therefore, to choose a solvent for which the partition coefficients are large so that the maximum separation is achieved. In paper chromatograms A_L/A_s is about 4 (Consden *et al.* 1944) so that α and β must be at least 10–15. This corresponds to an R_F value of 0.2–0.3.

In practice it is not always easy to find a two-component solvent (water is always one component) which will give R_F values of about 0.2 for a particular pair of sugars. Three-component solvents are, however, much more flexible in this respect. Originally these solvents were introduced in order to obtain a more convenient spread of R_F values for pentoses and hexoses than was possible with two-component solvents, but they can be readily applied to the separation of sugars with similar R_F values. The R_F value of a sugar can be altered by changing the amount of water dissolved in the organic phase; the general relationship between the amount of water in the organic phase and the R_F value is clearly shown by the figures in Table 1. The addition to a

two-component system of a third substance, which is readily soluble in both phases, increases the amount of water held in the non-aqueous phase, whereas addition of a substance which is soluble only in one phase diminishes the amount of water. Thus, by using the organic phase from a mixture consisting of two organic solvents and water, one of the organic solvents being miscible with water and the other immiscible, it is possible to alter the R_F value within wide limits. The R_F value for any particular pair of sugars can, therefore, be adjusted to about 0.2.

The apparatus used in the present experiments has the advantage that during the development of the chromatogram the phases on the paper are not disturbed. In the method described by Partridge (1948) the lid of the chamber is removed when the trough is filled with liquid, and the atmosphere is, therefore, initially unsaturated, and requires several hours at least to reach equilibrium with the solvent. During this time the advancing solvent front is losing solvent to the atmosphere, and particularly in the case of three-component systems may not have the same composition as the solvent in the trough. In extreme cases with certain mixtures the two phases become miscible and then all the sugars travel down the paper just behind the solvent front. By filling the trough without opening the chamber after the paper and atmosphere have reached equilibrium with the solvent, we can avoid any disturbance of the phases on the paper.

SUMMARY

1. A number of solvents have been examined for the separation of complex sugar mixtures on the paper chromatogram. It has been shown that two three-component mixtures, ethyl acetate-acetic acid-water and ethyl acetate-pyridine-water are more satisfactory than any previously described solvents for the qualitative and quantitative determination of the sugars in mixtures of the simple hexoses and pentoses.

2. Some of the factors which influence the separation of the sugars on the paper chromatogram are described and analyzed. It is shown that improved separations can be obtained by using solvents which give low R_F values, the solvent front being allowed to run off the paper. The use of a solvent with a low viscosity shortens the time needed for analysis because the solvent flows more rapidly through the paper.

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Zinc Uptake by *Neurospora*

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Among the minor constituents of living tissues zinc is one of great interest (see Lehman, 1939; Broh-Kahn & Mirsky, 1948; Warburg, 1948; Bertrand, 1948). It constitutes 0.33 % of carbonic anhydrase (Keilin & Mann, 1940), and it is a constituent of uricase which contains 1.3 % of zinc (Davidson, 1938; Holmberg, 1939) and possibly of other enzymes. It was found to be essential for growth and citric acid formation of *Aspergillus niger* (Bernhauer, 1928), for growth and fumaric acid production of *Rhizopus nigricans* (Foster & Waksman, 1939) and for growth and subtilin formation by *Bacillus subtilis* (Feeney, Lightbody & Garibaldi, 1947).

The presence of 0.2 mg. of zinc in the food of mice is stated by Bertrand (1948) to prolong the life of the animals from 2–3 weeks to 2–2.5 months.

Wassiljew (1935) demonstrated that the effect of zinc was not the same for biochemically different strains of *A. niger*, and he went so far as to suggest a characterization of *A. niger* strains based on their reactivity to zinc. Foster & Waksman (1939) interpret the function of zinc as catalyzing a more complete destruction of the glucose molecule with a consequent greater efficiency of energy utilization by the fungus.

In connexion with studies on the growth of *Neurospora crassa* we determined the amount of zinc necessary to obtain a maximum growth of that mould.

METHODS

Throughout the experiments mycelium from one spore was used. This was produced by crossing the two wild-type strains, nos. 25a and 1A of Beadle (1945). The culture solution was that described by Ryan, Beadle & Tatum (1943); it was autoclaved, and the cultures were treated under sterile conditions. Inocula were taken from cultures 4–5 days old.

Labelled zinc as chloride and varying amounts of non-radioactive zinc as sulphate were added to culture solutions at the start of the experiments. From the specific activities of the zinc present in the culture solution and in the mould, and the amount of zinc added to the culture solution, the zinc content of *Neurospora crassa* was calculated. We are much indebted to the Atomic Energy Commission of the U.S.A. and the Chief of the Isotope Branch, Dr Poul Aebersold, for supplying the zinc containing the radioactive isotope ^{65}Zn .

The activity of the ^{65}Zn taken up by *Neurospora* was less than 1 μcurie , and thus the radiation emitted could hardly produce any noxious action on the mould.

After centrifuging and thorough washing the *Neurospora* was dried at 105°, brought into solution by wet ashing, 5 mg. zinc as sulphate added as carrier, and the zinc precipitated with 8-hydroxyquinoline. The precipitate was filtered through a perforated aluminium dish covered by a layer of filter paper, and the dish placed under a Geiger-Müller counter of the type described by Madsen (1945). A sample of the active ZnCl_2 solution was treated in the same way. By comparing the activities of this 'standard' precipitate of known zinc content and that of the above-mentioned precipitates, the percentage of the zinc originally present in the culture solution which accumulated in the mould was calculated.

RESULTS

The results of some of our experiments, in which each *Neurospora* was grown in 150 ml. culture solution of varying zinc content for 5 days at 25°, are given in Table 1. This table and Fig. 1 show that the presence of 1 part of zinc in about 30,000 parts of dry mould is sufficient for an almost maximal growth, and the presence of 12.5 $\mu\text{g.}$ zinc in 150 ml. culture solution suffices to give that zinc content. It is advisable to add a somewhat larger amount of zinc to the culture solution, e.g. 200 $\mu\text{g./l.}$ as recommended by Ryan *et al.* (1943) or 450 $\mu\text{g./l.}$ as stated by Mitchell & Houlakan (1946).