CXXXIV. THE ANTISCORBUTIC FRACTION OF LEMON JUICE. IV.

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It was shown in the preceding communications of this series [Zilva, 1924, 1, 2; 1925] that concentrated antiscorbutic fractions could be obtained from decitrated lemon juice by removing from it sugar and substances precipitable by alcohol and by neutral lead acetate and eventually precipitating from the filtrate the active principle with basic lead acetate. Such an active fraction contains only about $\frac{1}{500}$ th of the dry matter of the original lemon juice but is undoubtedly a mixture. It was thought that it would be instructive at this stage of the inquiry to gain information about the behaviour of some of the inorganic constituents of lemon juice in the process of fractionation. Attention was mainly directed to the original juice, to the decitrated juice and to the purest fraction, *i.e.* the basic lead acetate fraction obtained after removal of the citric acid and impurities precipitable by alcohol and by neutral lead acetate. The sugar was not removed by fermentation previous to precipitation and the small amount which was brought down in this fraction by the basic lead acetate was ignored, as its removal by fermentation would most probably have been attended by the introduction of some of the inorganic constituents of the yeast.

The purest fraction was obtained by treating the decitrated lemon juice with neutral lead acetate, and centrifuging. Without removing the excess of neutral lead acetate, the supernatant fluid was then precipitated with basic lead acetate and centrifuged again. The precipitate which contained the active principle was dissolved in acetic acid, decomposed with magnesium sulphate, treated with two to three volumes of absolute alcohol, filtered and evaporated *in vacuo* to a small volume in order to remove the alcohol. In a few cases, mostly in the early experiments of this investigation, the basic lead acetate precipitate was decomposed with hydrogen sulphide. This method of removing the lead was discarded since it is uncertain whether the antiscorbutic factor withstands such treatment. As was already pointed out by one of us [Zilva, 1924, 2], test guinea-pigs do not thrive on fractions previously decomposed with hydrogen sulphide owing, most probably, to the toxic action of traces of mercaptan formed during the decomposition. On the other hand, the removal of the lead

by magnesium sulphate was found to be suitable for the biological tests of the antiscorbutic factor and it was, therefore, desirable to follow the technique which proved to yield active preparations.

Total ash.

As one would expect, the inorganic contents of lemon juice and the different elements constituting the ash vary within certain limits. Thus Oliveri and Gerrieri [1895] found *Citrus Limonum* L. to contain 0.20 % of ash, whilst Colby and Dyer [1890, 1891-2, 1893] found the juice from the Californian lemon to contain 0.63 %, 0.54 % and 0.78 % of inorganic constituents.

The following table gives five representative analyses of the ash of lemon juice as quoted by König:

Table I.

No.	Fe ₂ O ₃ —Al ₂ O ₃ %		CaO %	MgO %	K ₂ O %	Na ₂ O %	P ₂ O ₅ %	SO ₃ %	SiO ₂ %	Cl %
1	1.32	3.28	15.79	5.10	58.18	—	10.89	2.63	2.31	0.50
2	0.28		34.07	4.00	45.33	2.03	10.19	2.74	0.54	0.51
3	0.58		25.67	4.80	61.20	1.50	12.0	2.94	0.68	0.27
4	1.36		36.07	5.58	30.05	3.0	19.63	2.48	0.85	0.27
5	(2.57)		8.71	4.45	46.94	2.53	5.32	2.00	0.91	0.96

NOTES. In no. 5 the Fe—Al content is given as the double phosphate. In nos. 2, 3 and 4 the Mn₂O₄ contents are 0.28, 0.28 and 0.71 % respectively.

We determined the total ash of lemon juice by straining the freshly expressed juice through muslin, evaporating 100 cc. of the liquid and incinerating. Six batches examined yielded the following figures:

0.1503 %, 0.2124 %, 0.1732 %, 0.2034 %, 0.1896 %, 0.2084 %.

The ash of the purest fraction obtained from each of the above juices was also estimated and found to be:

0.00262 %, 0.00069 %, 0.00252 %, 0.00790 %, 0.00601 % and 0.002742 %.

These figures are no doubt higher than they should have been since the ash contained traces of the inorganic reagents used in the fractionation, such as calcium from the calcium carbonate employed in the decitration of the lemon juice. The lead acetate precipitates were in this case decomposed with hydrogen sulphide in order to obviate the introduction of minute quantities of magnesium. The figures, however, give us an idea of the order of the inorganic contents of the purest fraction.

The ash was examined for nickel, cobalt, boron, iodine, phosphorus, of which the first three were found to be absent from the purest fraction in quantities within the limits of the tests employed.

Nickel.

Tchugaeff's [1905] test was employed in detecting this element. A litre of lemon juice was fractionated as previously described and incinerated. The ash was taken up in dilute nitric acid and concentrated to 3 cc. Sufficient ammonium hydroxide was added to make the solution alkaline, after which

it was treated with 1 cc. of 1 % dimethylglyoxime solution, cooled and shaken with ether. The ethereal extract was colourless, nor was there a coloured zone between the aqueous and ethereal phases. Similar negative results were obtained with such preparations on two other occasions.

This test is claimed to be sensitive to 1 part in 400,000, yielding a pink-coloured nickel complex which rises, on shaking with ether, in a layer between the two liquids. On applying the test to a solution containing about $\frac{1}{30}$ th mg. nickel as nickel sulphate in presence of cobalt nitrate and copper sulphate of similar concentrations, a very marked positive result was obtained.

Cobalt.

The purest fractions of three different litres of lemon juice were tested for cobalt, utilising Vogel's test [1879] for this element. The incinerated material was extracted with dilute nitric acid and made up to 10 cc. To this were added 15 cc. of concentrated ammonium acetate solution, 1 cc. of 5 % acetic acid and 5 cc. of saturated ammonium thiocyanate. This mixture was shaken with a little acetone, and a mixture of alcohol and ether (1 : 10). No coloration was produced.

In the presence of cobalt a blue coloration is formed in the ethereal layer. A control solution containing $\frac{1}{30}$ th mg. of cobalt as cobalt nitrate in the presence of small quantities of copper sulphate and nickel sulphate, and ferric chloride gave a marked positive reaction.

Boron.

A preparation of the purest fraction equivalent to a litre of the original juice was evaporated to dryness at 105°. The residue was fused in a platinum crucible with four times its weight of sodium carbonate and the melt extracted with hot water and filtered. The filtrate was concentrated on the water-bath, acidified with hydrochloric acid and evaporated to dryness in the presence of a few drops of an alcoholic extract of turmeric. No coloration resulted. Only one batch equivalent to a litre of lemon juice was tested for boron. The test is claimed to be sensitive to $\frac{1}{800}$ th mg. A blank containing $\frac{1}{80}$ th mg. gave a positive reaction.

Iodine.

The method employed was Kelly and Husband's modification [1924] of Kendall's method. By this method the substance is fused with sodium hydroxide and the organic matter destroyed by potassium nitrate. The iodine which is fixed as sodium iodide is oxidised by bromine to iodic acid, liberated with potassium iodide and titrated with *N*/200 thiosulphate, using starch as the indicator.

The estimations were carried out on batches of the purest fraction equivalent to 1 litre of the lemon juice. The preparations were evaporated to dryness, and the residues treated according to the directions given by Kelly and Husband.

Four batches of the purest fraction equivalent to 1 litre of the original juice were examined separately, yielding 0.206 mg., 0.118 mg., 0.147 mg. and 0.118 mg. of iodine per batch. As the absolute limit of accuracy of the method employed is 0.1 mg. it may be concluded that the iodine content is 0.1–0.2 mg. per litre.

A blank experiment gave no coloration with starch.

It was previously shown [Zilva and Miura, 1921; Connell and Zilva, 1924] that the antiscorbutic factor did not dialyse to any appreciable extent in 3 days through collodion thimbles soaked in 88 % alcohol for 24 hours, whilst it did so through thimbles soaked in 95 % alcohol. Experiments were instituted to study the diffusibility of the iodine in antiscorbutic fractions as compared with that of simple inorganic iodides. Decitrated lemon juice was used as the source for the antiscorbutic vitamin and was dialysed in batches of 250 cc. contained in five thimbles of about 60–70 cc. capacity. The thimbles were closed with rubber stoppers and suspended by means of glass hooks fitted in the rubber. The dialysis, which lasted 72 hours, was carried out in distilled water which was changed every 2½ hours during the day but not at night. At the end of this period the thimbles were emptied and well washed. The contents were then concentrated, incinerated and the iodine estimated as above. As will be seen from Table II, the iodine can have no relation to the active principle. It did not diffuse even through a 95 % thimble which permits the free passage of the antiscorbutic factor.

Methylene blue, Bismarck brown and neutral red diffused through the thimbles soaked in 95 % alcohol but not through those soaked in 88 % alcohol.

Table II.

Thimble index	Potassium iodide added (mg.)	Iodine (mg.)
Not dialysed	—	0.1180
88 %	—	0.1180
88	4*	3.14
95	—	0.1475
95	4	0.1770

* i.e. 3.06 mg. iodine.

Phosphorus.

This element was determined by Neumann's method. It will be seen from Table III that the phosphorus content of the purest fraction is of the same order as that previously observed [Zilva, 1924, 2].

Table III. *Percentage of P.*

Raw juice	Basic lead fraction	Neutral lead fraction
0.00850	0.000517	0.000104
0.00700	0.000475	0.000115
0.00892	0.000554	0.000141
0.00806	0.000905	0.000110
0.00900	—	—

The various fractions were also examined for manganese and iron but some difficulties arose in connection with these tests and the matter is being investigated further.

CONCLUSIONS.

It is difficult to tell to what extent the figures obtained in this investigation are representative of lemon juice in general. One would naturally expect a certain amount of variation in the absolute values from juices of various origin and seasons. It is, however, doubtful whether the relative figures for the various constituents in the process of fractionation would be affected to any extent by these factors.

In the case of the elements not detected, it may safely be said that, unless it be in quantities which fall outside the limits of detection by the methods employed, they can have no bearing on the antiscorbutic activity of the juice.

Iodine may also be excluded in this sense since none of it diffuses through a collodion membrane soaked in 95 % alcohol, which indicates that it is present in a molecule much larger than that of the active principle. No appreciable quantities of the latter can be demonstrated in active solutions dialysed under the same conditions. This presence of iodine in a colloidal or semi-colloidal condition in lemon juice is, however, of interest.

SUMMARY.

1. The behaviour of the inorganic constituents of lemon juice in the various stages of the preparation of concentrated antiscorbutic fractions has been studied.

2. Nickel, cobalt and boron were found to be absent; phosphorus and iodine were found to be present in the purest fractions.

3. The iodine present in the purest fractions is present in a compound of a colloidal or semi-colloidal nature, and is separable from the active principle by diffusion.

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