

The Determination of Biphenyl and 2-Hydroxybiphenyl in Citrus Fruits

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A critical evaluation is made of the methods of analysis for biphenyl and 2-hydroxybiphenyl in citrus fruits as given in the recent "Preservatives in Food Regulations" 1974 and 1975. Recommendations for improvements in the statutory methods are made and an alternative G.L.C. procedure for both compounds is proposed. Typical levels found in samples of fruit and the recoveries obtained using both methods are given.

Biphenyl and 2-hydroxybiphenyl are used as fungicides in the post-harvest treatment of citrus fruits to prevent deterioration during transport and storage. The limits given in the "Preservatives in Food Regulations" 1974 and 1975 (Statutory Instruments No. 1119 and 1487) for residual levels are:

biphenyl	: 70 mg/kg,
2-hydroxybiphenyl	: 12 mg/kg.

Following the receipt by the Ministry of Agriculture, Fisheries and Food of some criticisms of the methods of analysis for these compounds (Schedule 5, Parts I, II and III), a thorough evaluation of the methods was undertaken at the Ministry's request. The methods are as described in an EEC directive¹ and have been translated into the Statutory Instruments (SIs) as required under the Treaty of Accession.

THE QUALITATIVE METHOD (SCHEDULE 5, PART I)

This was tested on several different samples, both spiked and unspiked, and was found to work satisfactorily; confirmation was obtained of the differing sensitivity of the method towards the two compounds; i.e. limits of detection of 5 mg/kg for biphenyl and 1 mg/kg for 2-hydroxybiphenyl.

THE CLEVENGER-TYPE SEPARATOR (SCHEDULE 6)

A description of this piece of apparatus was given in the EEC directive¹ and was put into the Statutory Instrument which came into force on 1 August 1974. It was not found possible to purchase the apparatus ready made from any of the major laboratory suppliers, and when it was constructed according to the dimensions given, the general appearance was considerably different from that in the diagram shown in the Statutory Instrument (Figure 1). Another piece was constructed following the same general appearance and size but disregarding the individual dimensions, and yet a third of our own design but following the same basic principles and overall size. It was found that the

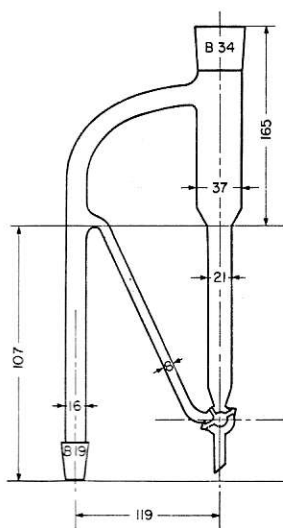


Fig. 1. Diagram of a modified Cleverger-type separator. Reproduced from Statutory Instrument 1975 No. 1487. Dimensions in millimetres.

recoveries of biphenyl and 2-hydroxybiphenyl were satisfactory using all three pieces of apparatus. It would appear, therefore, that minor deviations from the stated dimensions are not critical. In order to satisfy the need for a ready made apparatus an extensive search was made of the catalogues of the main laboratory suppliers. It revealed that a Type 2 receiver, 12.5 ml, as specified in BS 756: 1952, Dean and Stark apparatus, was the nearest item obtainable ready made and this could be purchased from most of the supply houses. This would only need the insertion of a three-way tap at the bottom of the graduated arm to be very similar in appearance and dimensions to the apparatus described in the Statutory Instrument. Such a modification could be easily made by a competent glass blower.

THE QUANTITATIVE METHOD FOR BIPHENYL (SCHEDULE 5, PART II)

In this method it was found that the two hour distillation period was satisfactory but that the analysis of the cyclohexane extract was time consuming and that very close attention to detail, particularly at the T.L.C. stage, was necessary before satisfactory results could be obtained. Table I shows the variation in absorbance of three standards each spotted on to three plates. Each group of three gives a reasonable straight line plot but the difference between each group is quite noticeable. Table II shows the variation of absorbance with the total time between spotting and measurement. Thus, the importance of a minimum but carefully controlled period of exposure is clearly demonstrated. The recoveries obtained using this method are shown as part of Table III. The specified method gave a limit of detection of 5 mg/kg. However, it was found that by viewing the developed plates under u.v. light instead of scraping off the spots this limit of detection could be lowered, in a semi-quantitative manner, to 1 mg/kg.

TABLE I
VARIATION IN ABSORBANCE OF STANDARD SOLUTIONS OF BIPHENYL
AFTER T.L.C.

$\mu\text{g BP}/10\text{ ml ethanol}$	Absorbance at 248 nm, 10 mm cell vs. blank		
	(a)	(b)	(c)
30	0.32	0.275	0.295
50	0.52	0.42	0.495
70	0.74	0.62	0.675

TABLE II
VARIATION IN ABSORBANCE OF STANDARD SOLUTIONS OF BIPHENYL WITH
TIME OF EXPOSURE ON T.L.C. PLATES

Time of exposure	Absorbance at 248 nm, 10 mm cell vs. blank
0-10 minutes	0.710
30 minutes	0.670
2 hours	0.655
3 hours	0.625
20 hours	0.040

TABLE III
RECOVERIES OF FUNGICIDES FROM CITRUS FRUITS

Fruit	Method	2-Hydroxybiphenyl			Biphenyl		
		Added, mg	Found, mg	Recovery, per cent.	Added, mg	Found, mg	Recovery, per cent.
Oranges	1 SI	—	(0.3)†	96	—	(4.8)	92
		2.3	2.5		5.0	9.2	
Oranges	2 G.L.C.	—	(0.3)	87	—	(18.4)	93
		3.0	2.9		15.0	32.3	
Oranges	3 SI	—	(0.1)	90	—	(6.5)	86
		1.0	1.0		8.0	13.4	
Grapefruit	1 SI	—	(0.1)	85	—	(4.7)	85
		2.0	1.8		10.0	13.2	
Grapefruit	2 G.L.C.	—	(0.2)	90	—	(0.3)	94
		3.0	2.9		15.0	14.4	
Lemons	1 SI	—	(0.1)	90	—	(<0.2)	98
		2.0	1.9		10.0	9.9	
Lemons	2 G.L.C.	—	(0.3)	90	—	(0.2)	99
		3.0	3.0		15.0	15.1	

† Figures in parentheses indicate natural values.

THE QUANTITATIVE METHOD FOR 2-HYDROXYBIPHENYL (PART III)

The chief problem in this method lies with the interpretation that might be put on the distillation instructions. Our findings were that if the instructions given

were followed, namely—"heating gently . . ., without allowing the purée to boil or foam"—then no 2-hydroxybiphenyl at all distilled over. It was necessary to boil the mixture moderately vigorously so that one drop distilled over every 3–5 seconds for a total period of six hours, before satisfactory recoveries were obtained. Using this distillation procedure and the specified spectrophotometric method with 4-aminophenazone, the overall recovery rate was confirmed to be about 80–90 per cent. Individual results are given in Table III. The limit of detection was governed by the value of the blank, which was equivalent to 0.7 mg/kg for 2-hydroxybiphenyl. Thus, the limit of detection is normally taken to be 1 mg/kg.

In view of the above difficulties with the statutory methods, a single G.L.C. method for both compounds was developed.

GLC PROCEDURE FOR BOTH BIPHENYL AND 2-HYDROXYBIPHENYL

A number of methods for the determination of these compounds by G.L.C. have been put forward in recent years. Morries² used a simple cold extraction of the comminuted fruit but gave no details of recoveries whilst his quoted limit of detection for 2-hydroxybiphenyl of 5 mg/kg is in our opinion too high when compared with the permitted level of 12 mg/kg. Beernaert³ used steam distillation for the separation stage, extracted the distillate with petroleum ether and then separated the two compounds from other components using gas chromatography with temperature programming. More recently, Westöö and Anderson⁴ have used a differently modified Clevenger trap⁵ for the steam distillation clean-up stage, with separate chromatographic columns for the determination of the two compounds. If an accurate result for low levels of 2-hydroxybiphenyl was required a further clean-up step was incorporated.

The method proposed here uses the modified Clevenger trap given in SIs 1119 and 1487 for the steam distillation step. This is considerably different in size and shape from that proposed by Westöö,⁵ and a single gas chromatographic column for the determination of both compounds using heptadecane as an internal standard. If, despite the fact that the amount of 2-hydroxybiphenyl found in the sample is well below the statutory limit, an accurate assessment of the amount present is required then it may be necessary to use the clean-up procedure recommended by Westöö and Anderson⁴.

Method

REAGENTS

1. *Sulphuric acid*, 50 per cent. v/v.
2. *Cyclohexane*, spectrosol grade.
3. *Heptadecane*, A.R. grade—dissolve 500 mg in cyclohexane and make up to 50 ml in a volumetric flask. 1 ml $\equiv$ 10 mg of heptadecane.
4. *2-Hydroxybiphenyl*, A.R. grade—dissolve 500 mg in cyclohexane and make up to 50 ml in a volumetric flask. 1 ml $\equiv$ 10 mg of 2-hydroxybiphenyl.
5. *Biphenyl*, A.R. grade—dissolve 500 mg in cyclohexane and make up to 50 ml in a volumetric flask. 1 ml $\equiv$ 10 mg of biphenyl.

APPARATUS

Food-mixer—Kenwood, or similar with liquidiser attachment.

Coffee grinder.

Distillation apparatus: 1 litre round-bottom flask fitted with a modified Clevenger separator (see Figure 1) and a water jacket condenser.

Heating mantle.

Vacuum evaporator with 50 ml flasks.

Gas chromatograph: Becker model 420 fitted with a flame ionisation detector and connected to a Servoscribe chart recorder, model RE541.

Column: glass, 2 m $\times$ 4.0 mm i.d., packed with 3 per cent. OV-17 on Gas Chrom. Q.

OPERATING CONDITIONS

Injector temperature: 210°C.

Column temperature—programme: 130°C for 10 min,
130–160°C at 10°C per min,
160°C for 22 min.

Detector temperature: 225°C.

Carrier gas flow rate: 30 ml nitrogen/min.

Detector gas pressures: hydrogen and air set according to manufacturer's instructions.

PROCEDURE

Separate the flesh and the peel from at least 2 kg of fruit. Cut the peel into small pieces and grind in a coffee grinder. Homogenise the flesh in a liquidiser. Combine the portions and mix well. Take 300 g of recombined fruit pulp and add 300 ml of water. Homogenise at low speed in a mixer and pour into the 1 litre round-bottom flask, rinsing the homogeniser with 50 ml water and adding this to the flask. Add an anti-foam tablet, a few anti-bumping granules and 15 ml of 50 per cent. sulphuric acid. Assemble the distillation apparatus and place 15 ml of water and 20 ml of cyclohexane in the side arm of the separator. Boil the mixture gently for 20 min and then more vigorously for the remainder of 2 hours such that one drop distils every 3–5 seconds. At the end of this time allow the mixture to cool. Run the water from the Clevenger separator into a 100 ml separating funnel and the cyclohexane into a 25 ml volumetric flask. Rinse the Clevenger separator with 4 ml of cyclohexane and add this to the water in the separating funnel. Shake well, allow the layers to separate, discard the water and add the cyclohexane to that in the 25 ml volumetric flask. Make up to volume with cyclohexane, add a little anhydrous sodium sulphate and mix well (this is extract "a"). Set up the separator again with a fresh portion of 20 ml of cyclohexane and repeat the two hour distillation and subsequent separation as above (this is extract "b").

PREPARATION OF STANDARD GRAPHS

Prepare five standard solutions (A-E) containing the indicated amounts of fungicide and internal standard:

		A	B	C	D	E
Volume (ml) of biphenyl solution	10 mg/ml	0.4	0.8	1.2	1.6	2.0
Volume (ml) of 2-hydroxybiphenyl	10 mg/ml	0.1	0.2	0.3	0.4	0.5
Volume (ml) of heptadecane	10 mg/ml	1.0	1.0	1.0	1.0	1.0

Make each up to 5 ml with cyclohexane.

Inject 1.5 μ l of each standard and record the chromatogram. Measure the areas (height $\times$ width at half height) of the peaks. Plot the area ratio of fungicide to internal standard against mg fungicide per 10 mg of heptadecane for each compound.

DETERMINATION OF BIPHENYL

To 10 ml of extract "a" add 0.4 ml of heptadecane solution, 10 mg/ml. Inject 1.5 μ l and record the chromatogram. Measure the peak areas and calculate the ratio of peak area of biphenyl to peak area of heptadecane. From the standard graph read off the biphenyl content, allowing for the amount of heptadecane added. If necessary the solution may be reduced in volume under vacuum but because of the volatility of biphenyl the volume should not be taken below 2 ml.

DETERMINATION OF 2-HYDROXYBIPHENYL

Combine 10 ml of extract "a" and 10 ml of extract "b" and add 0.1 ml of heptadecane solution, 10 mg/ml. Evaporate the solution under vacuum but without rotating the flask until the volume is reduced to a few ml. Inject 1.5 μ l and record the chromatogram. Measure the peak areas and calculate the ratio of peak area of 2-hydroxybiphenyl to peak area of heptadecane. From the standard graph read off the 2-hydroxybiphenyl content allowing for the amount of heptadecane added. The attenuation of the instrument should be adjusted so that the heptadecane peak is at least 40 per cent. of maximum. The biphenyl peak may then be off scale. Care should be taken to identify correctly the 2-hydroxybiphenyl peak particularly if it is small; natural substances present in citrus fruit sometimes give peaks corresponding to 0.5-1.0 mg/kg of 2-hydroxybiphenyl at the same retention time. If the main interest is in whether the fruit complies with the legal requirements this is unlikely to be serious but if an absolute value of 2-hydroxybiphenyl in the sample is required then the clean-up procedure, according to Westöo and Anderson,⁴ is employed.

Results and Discussion

Samples of oranges, lemons and grapefruits were examined for both fungicides by the quantitative methods given in the SI and also by the G.L.C. procedure.

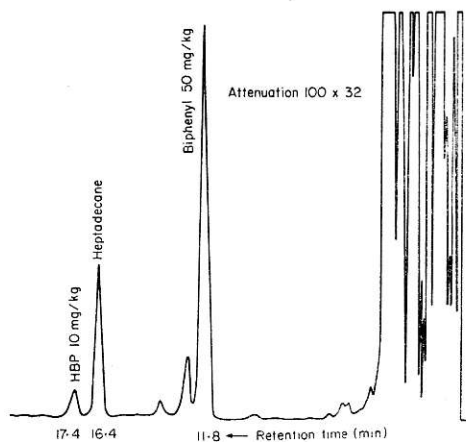


Fig. 2. Determination of biphenyl in oranges.

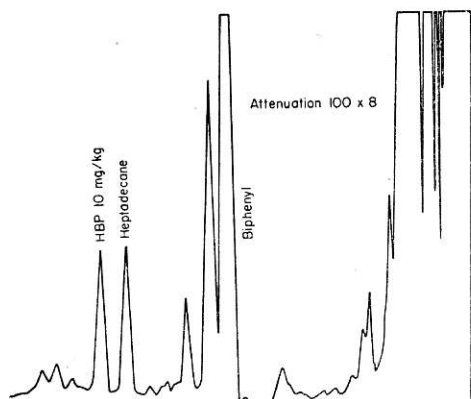


Fig. 3. Determination of 2-hydroxybiphenyl in oranges.

The recoveries obtained by a comparison of spiked and unspiked samples are given in Table III whilst the residues found in other samples using both methods are given in Table IV. The chromatograms shown in Figures 2 and 3 are those

TABLE IV
RESIDUES OF FUNGICIDES IN CITRUS FRUITS

Fruit		2-Hydroxybiphenyl, mg/kg		Biphenyl, mg/kg	
		By SI	By G.L.C.	By SI	By G.L.C.
Oranges	1	<1	0.5	35.4	39.0
	2	2.6	not determined	6.9	5.9
	3			5.7	6.2
Grapefruit	1	<1	0.3	5.3	5.5
	2			4.1	3.8
Lemons	1	<1	0.3	<0.2	0.2
	2			<0.2	0.2

of two 300 g portions from the same original sample of oranges spiked with 15 mg of biphenyl (50 mg/kg) and 3 mg of 2-hydroxybiphenyl (10 mg/kg) and then treated by the G.L.C. procedures given above. Figures 4(a) and (b) show the chromatograms of unspiked orange samples, with and without the clean-up step recommended for accurate low level determinations. After clean-up it can be seen that most of the spurious, and possibly interfering, peaks have been eliminated. Figure 4(c) shows the chromatogram of a sample of oranges, which was spiked with 3 mg/kg of 2-hydroxybiphenyl, and for which this clean-up procedure was used.

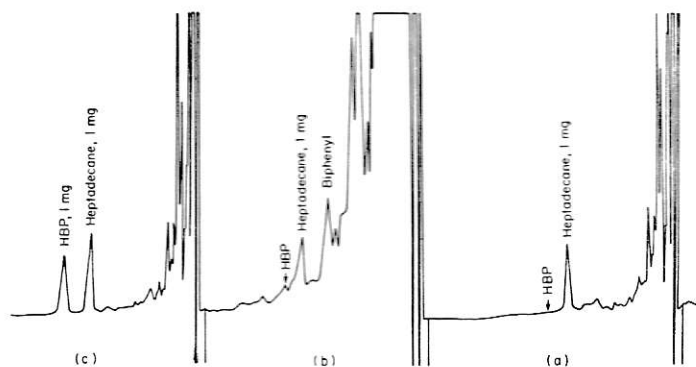


Fig. 4. Effect of clean-up on extracts of 2-hydroxybiphenyl from oranges. (a) Unspiked, after clean-up; (b) unspiked, before clean-up; (c) spiked, after clean-up.

Conclusion

A summary of our findings is that for accuracy the G.L.C. method is preferred for biphenyl and the Statutory Instrument method for 2-hydroxybiphenyl. However, for uniformity, simplicity and speed the G.L.C. method is preferred for both; the accuracy of this method for the low level determination of 2-hydroxybiphenyl can be improved by the incorporation of an additional clean-up stage.

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