SCHEDULE 5

ANALYSIS OF CITRUS FRUIT TREATED WITH BIPHENYL, 2-HYDROXYBIPHENYL OR SODIUM BIPHENYL-2-YL OXIDE

PART II

QUANTITATIVE ANALYSIS OF THE RESIDUES OF BIPHENYL IN CITRUS FRUIT

Purpose and scope

1. The method described below gives a quantitative analysis of the residues of biphenyl in whole citrus fruit. The accuracy of the method is ±10 per centum for a biphenyl content greater than 10 mg. per kg. of fruit.

Principle

2. After distillation in an acid medium and extraction by cyclohexane, the extract is subjected to thin layer chromatography on silica gel. The chromatogram is developed and the biphenyl is eluted and determined spectrophotometrically at 248 nm.

Reagents

3. The following reagents shall be used–

(a) concentrated sulphuric acid solution;

(b) silicone-based anti-foaming emulsion;

(c) cyclohexane (analytical reagent grade);

(d) hexane (analytical reagent grade);

(e) ethanol (analytical reagent grade);

(f) anhydrous sodium sulphate;

(g) silica gel GF 254 (Merck or equivalent);

(h) standard 1 per centum (weight/volume) solution of pure biphenyl in cyclohexane: dilute with cyclohexane to obtain the following three solutions–

(i) 0.6 µg/µl;

(ii) 1 µg/µl;

(iii) 1.4 µg/µl.

Apparatus

4. The following apparatus shall be used–

(a) a 1 litre mixer;

(b) a 2 litre distillation flask with a modified Clevenger-type separator as shown in the diagram in Schedule 6 and a cooled reflux condenser;

(c) a 10 ml. graduated flask;

(d) 50 µl. micropipettes;

(e) a thin layer chromatographic apparatus with 20×20 cm. plates;

(f) an oven;

(g) a centrifuge with 15 ml. conical tubes;

(h) an ultra-violet spectrophotometer.

Method of Analysis

5. The analysis shall be carried out as follows–

(a) Preparation and extraction: All the fruit in the sample for analysis is cut in half. Half of each piece of fruit is kept for qualitative analysis for residues of biphenyl, 2-hydroxybiphenyl or sodium biphenyl-2-yl oxide. The other halves are put all together and shredded in a mill or crushed until a homogeneous mixture is obtained. From this at least two sub-samples of 200 g. are taken for analysis in the following manner. Each sub-sample is placed in a mixer with 100 ml. of water and mixed at slow speed for several seconds. Water is added until the volume of the mixture reaches ¾ of the capacity of the mixer, and the mixture is then mixed for 5 minutes at full speed. The resulting purée is transferred to the 2 litre distillation flask. The mixer is rinsed with water and the rinsings added to the contents of the flask. (The total quantity of water to be used in mixing and rinsing is 1 litre.) To the mixture are added 2 ml. sulphuric acid, 1 ml. anti-foaming emulsion and several anti-bumping granules. The separator and reflux condenser are fitted on to the flask. Distilled water is poured into the separator until the water level is well past the lower arm of the lateral return tube, followed by 7 ml. cyclohexane. Distillation is carried out for about 2 hours. The lower aqueous layer in the separator is discarded and the upper layer is collected in the 10 ml. graduated flask. The separator is rinsed with about 1.5 ml. of cyclohexane and the rinsings added to the contents of the flask, which are then brought up to volume with cyclohexane. Finally a little anhydrous sodium sulphate is added and the mixture is shaken.

(b) Chromatography: 30 g. of silica gel and 60 ml. of water are placed in a mixer and mixed for one minute. The mixture is then spread on to 5 chromatographic plates to form a layer approximately 0.25 mm. thick. The plates covered with this layer are subjected to a stream of hot air for 15 minutes and then placed in an oven where they are kept for 30 minutes at a temperature of 110°C. After cooling, the surface layer of each plate is divided into 4 lanes, 4.5 cm. wide, by parallel lines penetrating the silica gel down to the surface of the glass plate. 50 µl. of the extract to be analysed are applied to one lane of each plate as a narrow band of contiguous spots approximately 1.5 cm. from the lower edge of the plate. 50 µl. of the standard solutions (i) (ii) and (iii), corresponding respectively to 30, 50 and 70 µg. levels of biphenyl are applied in the same way to the three remaining lanes, one solution to each lane.

If a large number of samples are being analysed at one time, standard solutions need not be applied to every plate. Reference may be made to a standard curve provided that this curve has been prepared from the average values obtained from 5 different plates to which the same standard solutions have been applied.

(c) Development of chromatograms and elution: The chromatograms are developed with hexane to a height of 17 cm. in tanks previously lined with filter paper. The plates are air dried. By illuminating the plates with ultra-violet light (254 nm.), the areas of silica gel containing biphenyl are located and marked off in rectangles of equal area.

The entire layer of silica gel within the areas thus marked off is immediately scraped from the plate with a spatula. The biphenyl is extracted by mixing the silica gel with 10 ml. of ethanol and shaking several times over a period of 10 minutes. The mixture is transferred to the centrifuge tubes and centrifuged for 5 minutes at 2,500 revolutions per minute.

A control sample of silica gel is taken by the same method using an area of the same size. If a series of analyses are made, this control area is taken from an unused lane of a plate

and below the solvent front; if a single analysis is made the control sample is taken from an area below one of the positions at which the standard biphenyl is located.

(d) Spectrophotometric determination: The supernatant liquid is decanted into the spectrophotometer cells and the absorption determined at 248 nm. against a control extract from a chromatographic area free from biphenyl.

Calculation of results

6. A standard curve is drawn, plotting the biphenyl values of 30, 50 and 70 µg. against the corresponding absorptions, as determined on the spectrophotometer. This gives a straight line which passes through the origin. This graph allows the biphenyl content of the samples to be read directly in mg. per kg. from the absorption value of their extracts.