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THE USE OF REFEREE SAMPLES TO TEST AND COMPARE THREE METHODS FOR ANALYSES OF BIPHENYL IN CITRUS FRUITS

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By

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THE USE OF REFEREE SAMPLES TO TEST AND COMPARE
THREE METHODS FOR ANALYSES OF
BIPHENYL IN CITRUS FRUIT

In order to compare the three methods for measuring the amount of biphenyl residues found in citrus fruits shipped in fiberboard containers treated with biphenyl, a number of known, or referee, samples of various citrus oils with known amounts of biphenyl in them were prepared. One lot was prepared by Dr. E. A. Beavens in the Fruit and Vegetable Laboratory of the U. S. Department of Agriculture in Pasadena, California, and the other lot was prepared by Dr. W. F. Newhall of the University of Florida Citrus Experiment Station at Lake Alfred, Florida. These samples were then analyzed at four places--at the Pasadena Laboratory, Lake Alfred Laboratory, at the Monsanto Central Research Laboratory in Dayton, Ohio and at The Institute of Paper Chemistry in Appleton, Wisconsin.

The results of these analyses are contained in Tables I and II.

It should be noted that at the time these referee samples were analyzed, the work on method development up to that time had been done using Valencia oranges. The data indicate the greatest uniformity of results in the referee samples containing Valencia orange oil.

It would appear that one of the causes for variation in results is in the stage of the method in which the "interfering" substances are oxidized by permanganate. Since this work was done additional refinements have been added to the oxidation process which have reduced the per cent of error, particularly in analyses of lemons and, to a lesser extent, grapefruit.

TABLE I

DATA FROM ANALYSES OF CALIFORNIA REFEREE SAMPLES

Base	Sample No.	mg./ml. Biphenyl Added	Florida (Infrared) Results	% Error of av.	Pasadena (Chromatostrip) Results	% Error of av.	I.P.C. (Ultraviolet) Results	% Error of av.
Valencia Orange Oil	1	1.53	1.52;1.58; 1.57 av. 1.556	1.7	1.55;1.64; av. 1.595	4.25	1.40;1.54; av. 1.47	3.92
	2	5.90	5.81;5.97; 5.76;5.67; av. 5.802	1.7	5.87;5.56; av. 5.715	3.14	5.70;5.00; av. 5.35	9.32
	3	11.60	11.89;11.61; 11.71 av. 11.736	1.17	11.6;11.7; av. 11.65	0.43	11.3;11.0; av. 11.15	3.88
Navel Orange Oil	1	7.40	7.30;7.23; 7.35 av. 7.293	1.45	6.6;7.65; av. 6.625	10.5	6.80;6.68 av. 6.74	8.9
	2	5.28	5.11;5.19; 5.13;5.20; av. 5.157	2.33	4.94;4.95; av. 4.945	6.34	5.10;4.95; av. 5.025	4.84
	3	27.13	21.98;21.38; 22.44 av. 21.93	22.8	26.0;24.5; av. 25.25	6.93	25.1;25.6; av. 25.35	6.56

TABLE I (Continued)

Base	Sample No.	mg./ml. Biphenyl Added	Florida (Infrared) Results	% Error of av.	Pasadena (Chromatostrip) Results	% Error of av.	I.P.C. (Ultraviolet) Results	% Error of Av.
Lemon Oil	1	6.24	5.68;5.80; av. 5.74	8.02	5.86;5.89; av. 5.875	5.85	6.10;6.35; av. 6.225	0.24
	2	22.78	22.48;21.52; 20.36;20.44; av. 20.20	11.3	22.79;22.08; av. 22.435	1.51	21.6;21.6 av. 21.6	5.18
	3	33.38	33.09;31.74; 32.24;31.76; av. 32.81	1.70	32.88;34.5 av. 33.69	0.93	28.0;29.4 av. 28.7	14.01
Grapefruit Oil	1	13.75	11.92;12.64; 11.74;11.24; av. 11.88	13.60	12.5;13.74; av. 13.12	4.58	11.6;12.9 13.6;13.3; av. 12.85	6.55
	2	40.82	36.92;36.64; 36.72 av. 36.76	9.94	38.12;37.9 av. 38.01	6.88	36.6;39.0 av. 37.8	7.39
	3	44.44	42.88;44.72; 43.15 av. 43.58	1.94	42.4;41.2; av. 41.8	5.95	40.4;39.8 av. 40.1	9.98

TABLE II

DATA FROM ANALYSES OF FLORIDA REFEREE SAMPLES

Base	Sample No.	mg./ml. Biphenyl Added	Florida Results	% Error of av.	Pasadena Results	% Error of av.	Monsanto Results	% Error of av.	I.P.C. Results	% Error of av.
Orange Oil	1	2.94	3.19;3.07 av. 3.13	6.46	2.71;2.98 av. 2.75	6.46	1.17; 1.09 av. 1.13	61.6	2.92;3.0 av. 2.96	0.67
	2	5.60	5.66;5.92 av. 5.79	3.39	5.29;5.39 av. 5.34	5.36	5.6;5.5 av. 5.55	0.89	5.45;5.45 5.35 av. 5.416	3.28
	3	23.5	22.7;24.0 av. 23.3	0.85	23.03;23.49 av. 23.26	1.02	22.7;24.4 av. 23.55	0.21	22.4;22.1 av. 21.8	5.95
Lime Oil	1	1.01	1.16;1.20 av. 1.18	16.8	1.17;1.11; 1.13;1.13 av.1.135	14.4	--	--	1.56;1.61 1.66 av. 1.61	50.5
	2	6.20	6.31;6.62 av. 6.46	4.20	5.93;5.91 av. 5.92	4.52	--	--	6.35;6.45; 6.45 av. 6.416	3.48
	3	29.5	30.4;28.9; av. 29.7	0.68	27.50;26.85 av. 27.175	7.9	28.8;28.0 av. 28.4	3.73	28.3;28.3; 27.8 av. 28.13	4.65

TABLE II (continued)

Base	Sample No.	mg./ml. Biphenyl Added	Florida Results	% Error of av.	Pasadena Results	% Error of av.	Monsanto Results	% Error of av.	I.P.C. Results	% Error of av.
Grapefruit Oil	1	0.76	0.76;0.82 av. 0.79	3.95	0.8;0.76 0.8;0.75 av. 0.77	1.31	0.94;0.85 av. 0.895	17.1	0.94;0.91; 0.95 av. 0.933	35.9
	2	6.25	6.19	0.96	5.85;5.75 av. 5.80	7.2	6.6;6.3 av. 6.45	3.2	5.78;5.78 5.65 av. 5.736	23.7
	3	29.4	30.5;29.9 av. 30.19	2.68	28.29; 27.19 av. 27.74	5.65	27.2;27.8 av. 27.5	6.46	26.6;27.8; av. 26.8	7.96

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FUNGICIDE DETERMINATION

Quantitative Determination of Biphenyl in Citrus Fruits And Fruit Products by Means of Chromatostrips

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Biphenyl-treated collars, liners, and pads are currently being used by the citrus industry to prevent molding during shipping and storage of citrus fruits. The possibility that the biphenyl vapors will be absorbed by the fruit has created a need for an analytical method for determining the amounts of biphenyl in the fruit and in processed citrus products. A procedure has been developed to separate the biphenyl from interfering citrus oils by chromatographing on chromatostrips. Two methods of quantitatively measuring the amount of biphenyl on the strips are presented. A visual method makes use of the point of minimum detection of the biphenyl spot under ultraviolet light. In the second method, the biphenyl spot is removed from the strip and eluted with alcohol. The concentration of biphenyl is then measured in an ultraviolet spectrophotometer at 248 mμ. Added biphenyl has been determined in citrus juices in as low a concentration as 0.1 p.p.m. and in citrus peel as high as 600 p.p.m. These methods are well suited to determine the amounts of biphenyl absorbed by citrus fruits during storage or shipment in a biphenyl atmosphere, and amounts occurring in processed citrus products.

THE USE OF BIPHENYL-TREATED COLLARS, liners, and pads for the prevention of molding during shipping and storage of citrus fruits in fiberboard cartons has created a need for an analytical method for the determination of this compound in juice, peel, whole fruit, and processed citrus products.

Tomkins and Isherwood's (*9*) colorimetric method does not have the accuracy required, because of the partial destruction of biphenyl by the sulfuric acid treatment. Steyn and Rosselet (*8*) used an ultraviolet absorption method by applying a correction factor for the absorption caused by the citrus oils. The correction factor was obtained from biphenyl-free fruit, but the accuracy of this method is open to question, as the ultraviolet absorption of orange oil varies with individual fruits (*2*). Knodel and Elvin (*5*) have developed an infrared method for the determination of biphenyl in treated fiberboard cartons where citrus oil is not a factor.

A rapid method is needed capable of detecting biphenyl in as small an amount as 1 p.p.m. in juice, peel, or whole fruit of all varieties of citrus fruits. To achieve this purpose, a chromatographic method was developed for separating the biphenyl from interfering citrus oils by using the chromatostrips of Kirchner, Miller, and Keller (*4*).

These are glass strips coated with an ultraviolet fluorescing adsorbent which is held in place by a suitable binder. The coated strips are used as a microchromatographic column in a manner analogous to paper chromatography, and after development of the chromatogram and removal of excess solvent the strips are examined under ultraviolet light to locate the biphenyl spot.

Two methods of measuring the amount of biphenyl in the spot are presented

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concentrations are applied to find the one giving the minimum detectable amount of biphenyl. It is rapid and useful for field work wherever an ultraviolet spectrophotometer is not available. In the other method, the chromatographed material is eluted and measured in an ultraviolet spectrophotometer at 248 mμ.

Equipment and Materials

Clevenger distillation apparatus (*10*) modified by replacing the regular 2-ml. receiving buret with a 10-ml. buret constructed from a 10-ml. pipet graduated in 0.1 ml.

Boiling flask, 3-liter, with standard-taper neck.

Electric heating mantle.

Pipets, 0.01-ml.

Volumetric flasks, 1-, 2-, and 3-ml.

Syringe microburet (optional for the visual method).

Ultraviolet lamp. Lamps with main output at 365 mμ are unsatisfactory; a short-wave-length lamp (253.7 mμ) is essential

Ultraviolet spectrophotometer

n-Heptane or iso-octane, free from aromatic and other ultraviolet-absorbing impurities.

Low-boiling (30° to 60° C.) petroleum ether, free of ultraviolet-absorbing impurities

Reagent-grade silicic acid.

Zinc-cadmium sulfide.

Zinc silicate.

Starch (Clinco 15 modified starch), or 2 to 1 mixture of ordinary cornstarch and Superior AA tapioca flour.

A mechanical blender.

Water-soluble grease (*7*).

Test tubes, 18 × 150 mm.

Mechanical draft oven.

Glass strips, 13 × 136 mm.

Citrus juice reamer, hand or mechanical.

Glass-stoppered flasks or bottles for oil samples, 10-ml.

Preparation of Materials

n-Heptane or iso-octane (pure grade) is further purified by fractional distillation, followed by chromatographic purification by the method of Graff and associates (*1*).

Petroleum ether (reagent grade) is purified in the same manner as the heptane or iso-octane.

Chromatostrips are prepared by the method originally published (*4*), except that either Clinco 15 modified starch or a mixture of cornstarch and tapioca flour is substituted for the Amioca starch.

Preparation of Sample

The sampling procedure varies. For juice, a 1500-ml. sample is taken. If it is desired to analyze a single fruit, it is ground with 200 ml. of water in a blender to a slurry which is then diluted to 1500 ml. To analyze peel, 100 grams of peel are ground to a slurry with 100 ml. of water and then diluted to 1500 ml.

The 1500-ml. sample is placed in the 3-Clevenger buret. (The regular 2-ml. Clevenger buret cannot be used, because with the amount of solvent used the condensed water tends to collect in drops and push the solvent over into the distilling flasks.) One milliliter of *n*-heptane or iso-octane is placed in the 10-ml. receiving buret, and the contents of the flask are gently boiled for 3 hours without foaming or bumping. After distillation, *the condenser and upper part of the still* head are rinsed with 1 ml. of the solvent, and the total volume of heptane or iso-octane solution, containing the citrus oil and the biphenyl, is read and drained into a flask.

Visual Method

Portions of the heptane-oil-biphenyl solution are diluted with *n*-heptane to make dilutions of 1 to 10 and 1 to 100. With a 0.01-ml. pipet, portions of the undiluted and diluted solutions are spotted at the origin on a series of chromatostrips. A syringe microburet (*6*) is convenient for spotting the strips and decreases the number of dilutions needed.

The strips are placed in test tubes containing 1 ml. of purified petroleum ether (boiling point 30° to 60° C.) and stoppered. When the solvent reaches (by *capillarity*) a *line of* 5 cm. above the origin (approximately 7 minutes), the strips are removed and the excess solvent is evaporated in the air (traces of solvent tend to obscure the ultraviolet-absorbing spot). The strips are then examined under the ultraviolet lamp. If biphenyl it will appear as a dark spot on the fluorescent background. A series of strips containing 0.01-ml. or multiples of 0.01-ml. portions of the several dilutions should be prepared in this manner. For example, from the original solution 0.005-, 0.01-, 0.02- and 0.05-ml. portions are spotted on individual strips; from the 1 to 10 dilution 0.005-, 0.01-, and 0.02-ml. portions are applied; and from the 1 to 100 dilution similar amounts are spotted. When amounts greater than 0.01 ml. are used, separate 0.01-ml. portions are applied on the strip and the solvent is removed by an air stream before the next 0.01 ml. is added.

After development, the strips are examined under ultraviolet light and the strip is selected which just shows a faint but definite dark biphenyl spot. For example, if 0.005, 0.01, 0.02, and 0.03 ml. of a given solution are spotted on strips and, after development, biphenyl spots are visible on all strips except the 0.005 one, then additional strips are run with amounts between 0.005 and 0.01 ml. to find the strip with the minimum detectable amount. In this laboratory this was found to be 0.55 γ of biphenyl. (As this may vary with individuals, each analyst should determine this value for himself.)

Calculation. Milligrams of biphenyl

$$= 5.5 \times 10^{-4} \frac{V}{MC}$$

where

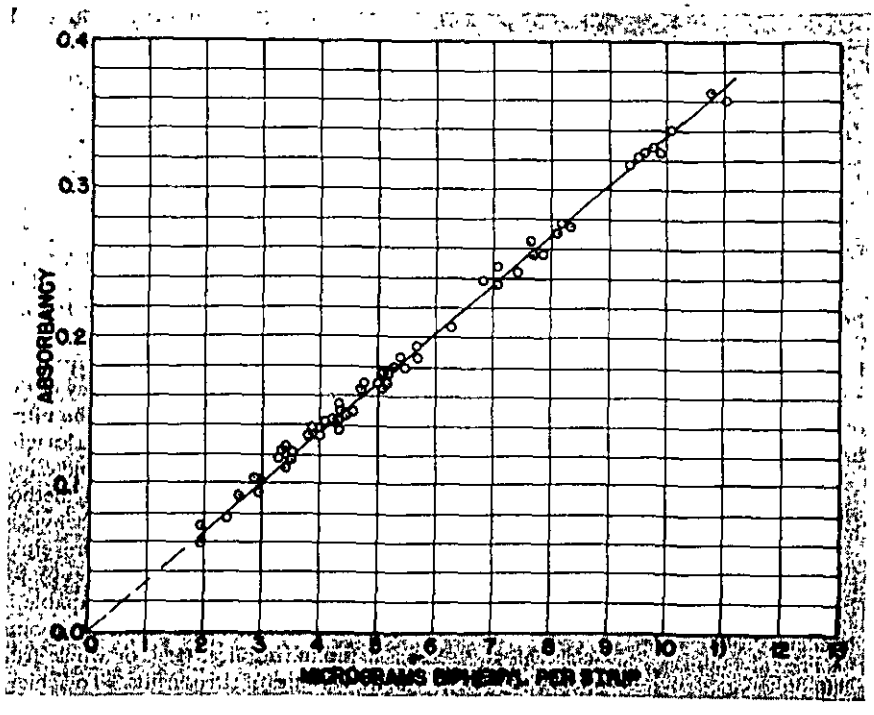
V = ml. of heptane or iso-octane solution in receiving buret

M = ml. of sample spotted on strip

C = dilution factor, 0.1, 0.01, etc.

Figure 1. Spectrophotometric standard curve for determination of biphenyl

λ = 248 mμ. 10 mm. cell



Spectrophotometric Method

In order to increase the accuracy of the method, a spectrophotometric procedure was adopted for measuring the amount of biphenyl after it is separated by chromatography on chromatostrips. In this case, prior to the application of the biphenyl solution to the strips, the latter are washed by placing in stoppered test tubes containing 1 ml. of 95% ethyl alcohol and allowing the solvent to travel up the strip. They are then dried at 85° C. for 4 minutes in a mechanical convection oven, and after cooling in a desiccator they are ready for use. This is done just prior to the analysis, in order to decrease the blank by removing impurities adsorbed from the air.

For the spectrophotometric method the biphenyl solution is applied to the chromatostrip by means of a syringe microburet (*6*), capable of delivering 0.1 microliter of solution. Both the ground-glass joint of the delivery tip and the plunger of the syringe are lubricated with a water-soluble grease (*7*) in order to avoid evaporation losses.

The strips are developed in petroleum ether (30° to 60° C.) which has been freed of ultraviolet-absorbing materials. In this case development is allowed to proceed until the solvent has traveled 10 cm., in order to get a greater separation of the biphenyl from the other ultraviolet-absorbing materials in the citrus oil. The strips are then carefully dried in an air stream until the solvent odor disappears, and under ultraviolet light the limits of the biphenyl spot are marked. These spots are then transferred to a sintered-glass funnel by scraping off a standard area 22 mm. long the full width of the strip, with the biphenyl spot located at the center of the area. To avoid transfer losses, this is best accomplished by use of a special cone-shaped funnel, 40 mm. in diameter at its widest part with a 10-mm. sintered-glass disk at the bottom. The biphenyl is eluted with 95% alcohol, the eluate being caught directly in a 3-ml. volumetric flask, which is a convenient volume for filling the 10-mm. quartz cells. After making up to volume, the ultraviolet absorption of the eluate is measured in the spectrophotometer at 248 mμ, using 95% alcohol as the blank. The reading is then corrected using a blank obtained by running several strips through the procedure without biphenyl prior to each day's run. The strips are run in triplicate for each analysis and the average density value is used. From the corrected density value the amount of biphenyl on the strip is determined from a standard curve (Figure 1).

Results and Discussion

These methods are based on the fact that the oxygenated constituents in citrus oil are tightly adsorbed at the origin when petroleum ether is used as the solvent (*3*). Biphenyl, a hydrocarbon, moves and has an R_f value of 0.45 (on the alcohol-washed strips), while the citrus oil hydrocarbons are less tightly adsorbed and move close to the top of the strip (R_f 0.95). In this chromatographic separation, therefore, the biphenyl is well separated from the other constituents of the sample and may be observed and eluted without interference.

For the visual method the minimum limit of detection on a strip has been found to be 0.55 γ of biphenyl, but the concentrating effect of steam distillation in the Clevenger apparatus permits 0.05 p.p.m. to be detected in citrus juice when a 1500-ml. sample is used, or 1.5 p.p.m. with a 50-ml. sample of juice from a single fruit. With 19 samples ranging from 0.5 to 575 p.p.m., the average error was found to be 11.3%. The tendency toward larger errors is increased at higher concentrations, because of the large dilution factors, which tend to amplify the errors in reading the minimum visible concentration. Table I shows the recovery of added biphenyl by the visual method.

For the recovery experiments in the juice and whole fruit, aliquots of a biphenyl-heptane solution were added to the sample, and for the peel samples weighed amounts of biphenyl were added directly to the ground peel.

Figure 1 shows the spectrophotometric standard curve, which was prepared by adding known amounts of biphenyl to various citrus samples. This curve includes determinations on lemons, limes, grapefruit, Valencia oranges, and navel oranges as peel, juice, and whole fruit samples to which varying amounts of biphenyl were added. It covers amounts as low as 0.1 p.p.m. in the juice up to 600 p.p.m. in peel samples. The average error is ±2.8% with a maximum error of 9.3% in 57 analyses. Of these 57 analyses only 7 were over 5% in error.

Table I. Recovery of Added Biphenyl from Citrus Products Determined by Visual Analysis

Product	Biphenyl Added, p.p.m.	Biphenyl Added, mg.	Biphenyl Found, mg.	Biphenyl Difference, mg.	Error, %
Valencia orange juice	0 5	0 75	0 68	0 07	9 3
	0 5	0.75	0.74	0 01	1 3
	1.0	1 50	1 60	0.10	6 7
	1.25	1 68	1 89	0.21	12 5
	10 0	15.0	15.6	0.60	4 0
Lemon juice	0 1	0.15	0 17	0.02	13 3
	0 5	0.75	0 64	0.11	14 7
	0.5	0.75	0.85	0 10	13 3
	1 0	1.50	1.60	0.10	6 7
	1 0	1.50	1 76	0 26	17 3
	1 1	1.65	1 57	0.08	4 8
	1.1	1.65	1.87	0 22	13 3
	2 0	3.00	3 18	0.18	6.0
	4 0	6.00	7.00	1 00	16 7
	5 0	7.50	6.48	1 02	13.6
	5.0	7.50	8 50	1 00	13 3
Grapefruit juice	1 08	1.62	1 38	0 24	14 8
Orange peel	567	56 7	71 5	14 8	26 1
Lemon peel	575	57.5	53 4	4 1	7 1

Acknowledgment

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of the phosphate caused no interference. More than this amount of fluoride inhibited the development of the niobium molybdenum blue color.

Nitrate present in the original solution is driven off on fuming. However, a study was made of the effect of nitrate, since Davydov, Vaisberg, and Burkser in their method for steels developed the color in a solution containing nitric acid. It was found in this laboratory that up to 0.3 gram of sodium nitrate added to the 50-ml. volumetric flask before the addition of the phosphate caused no interference, if the color were read within 3 minutes. After this interval of time the color faded rapidly.

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Infrared Determination of Biphenyl in Citrus Fruits

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MUCH interest has arisen concerning the biphenyl residues remaining in the peel or juice of citrus fruit packed in cartons impregnated with Phenodor X (a mixture of biphenyl, petroleum wax, and odor counteractants) or similar fungistatic preparations containing biphenyl. Several quantitative analytical methods for determining biphenyl in citrus peel and juice have been described. Tomkins and Isherwood (*4*) developed a method whereby a sample of biphenyl in orange oil, obtained by steam distillation of minced peel or pulp, was extracted with concentrated sulfuric acid to remove the orange oil. The biphenyl was then determined colorimetrically. The error in this method was reported as ±20%.

In the biphenyl analysis of fruit developed by Steyn and Rosselet (*3*), the biphenyl content of steam-distilled oil was calculated from the ultraviolet absorption at 250 mμ. A correction for the orange oil present was applied, based on the absorption of the oil at 375 mμ, at which wave length biphenyl does not absorb. Some difficulty has been encountered by other workers in obtaining reproducible results using the method of Steyn and Rosselet. This difficulty is associated with the correction for the orange oil present.

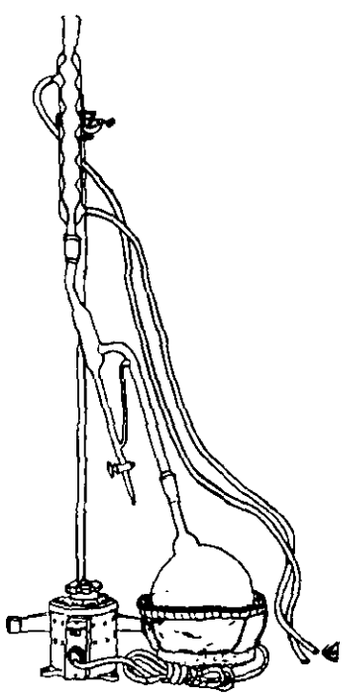

Figure 1. Liquid-Liquid Extractor

A quantitative determination of the biphenyl content of fiberboard cartons impregnated with Phenodor X has been developed by Knodel and Elvin (*2*). In this method biphenyl is extracted from a fiberboard sample with carbon tetrachloride and the absorption peak at 14.34 microns in the infrared region is measured. The mean per cent error of this method is 1.9%.

A quantitative analytical method has been devised for the direct determination of biphenyl in orange oil obtained by steam distillation of minced peel or juice. Use is made of the biphenyl absorption peak at 14.34 microns in the infrared. Variations in the optical properties of individual orange oil samples do not affect the accuracy of the results when calculated using a base-line technique. This ability to use orange oil as the solvent greatly simplifies the isolation and determination of biphenyl in fruit.

EQUIPMENT

For the analysis, a Beckman Model IR-2 spectrophotometer with rock salt optics is used. It is equipped with a synchronous wave-length drive motor and a chart recorder. The absorption cells used have rock salt windows separated by either a 0.1- or 0.4-mm. spacer. (The first analyses were made using a 0.1-mm. spacer. However, the standard curves in Figure 3 show that a 0.4-mm. spacer gives better resolving power, especially for dilute solutions of biphenyl in orange oil, and this spacer is now used for all analyses.)

REAGENT

Standard solutions are prepared by dissolving known amounts of pure biphenyl (melting point 68.5° to 69.5° C.) in technical

Table 1. Recovery of Biphenyl Using Liquid-Liquid Extractor

No.	Biphenyl Added P.p.m.	Biphenyl Added Mg.	Biphenyl Found, Mg.	Recovery, %
One Half Whole Fruit (Approximately 130 Grams)				
1	90	11.7	11.9	102
2	123	16.0	16.6	103
3	65	8.4	8.2	98
4	54	7.0	6.7	96
5	47	6.1	6.1	100
6	58	7.6	7.7	101
7	40	5.2	4.8	93
8	42	5.4	5.3	98
9	36	4.7	4.5	96
10	77	10.0	9.90	99
11	77	10.0	10.31	103
12	114	14.77	13.97	95
13	114	14.77	13.34	90
14	38	4.98	4.52	91
15	77	10.0	9.73	97
16	77	10.0	9.99	100
17	114	14.77	13.81	94
18	114	14.77	13.80	94
19	38	4.98	5.00	100
20	38	4.98	4.46	90
Valencia Juice (Approximately 1600 Grams)				
21	3.1	4.98	4.63	93
22	3.1	4.98	4.98	100
23	3.1	4.98	5.08	102
24	3.1	4.98	4.96	100
25	1.8	2.85	2.96	104
26	1.8	2.85	2.87	101
27	1.8	2.85	2.95	103
28	1.0	1.60	1.68	105
29	1.0	1.60	1.60	100
30	1.0	1.60	1.65	103
31	1.0	1.60	1.54	96

Average recovery 98%.
Standard deviation ±4.24%.

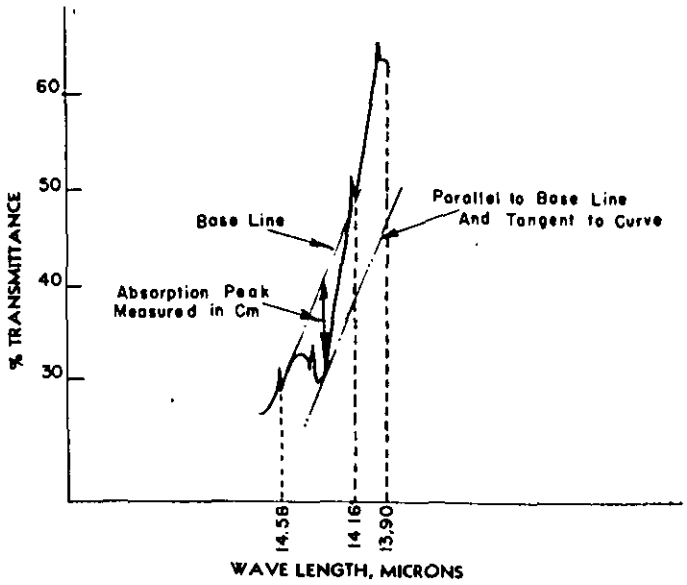

Figure 2. Infrared Chart Showing Base-Line Technique for Measuring Absorption Peak

grade *d*-limonene. Both reagents were obtained from the Matheson Co.

PROCEDURE

The isolation procedure originally used comprised weighing 20 to 25 whole oranges to the nearest gram, grinding them, and performing a conventional type steam distillation. This method requires rather elaborate equipment and constant attention to minimize entrainment and to regulate the rate of distillation. *Routine isolation of biphenyl* is more conveniently accomplished using a small sample and performing the steam distillation by refluxing the sample.

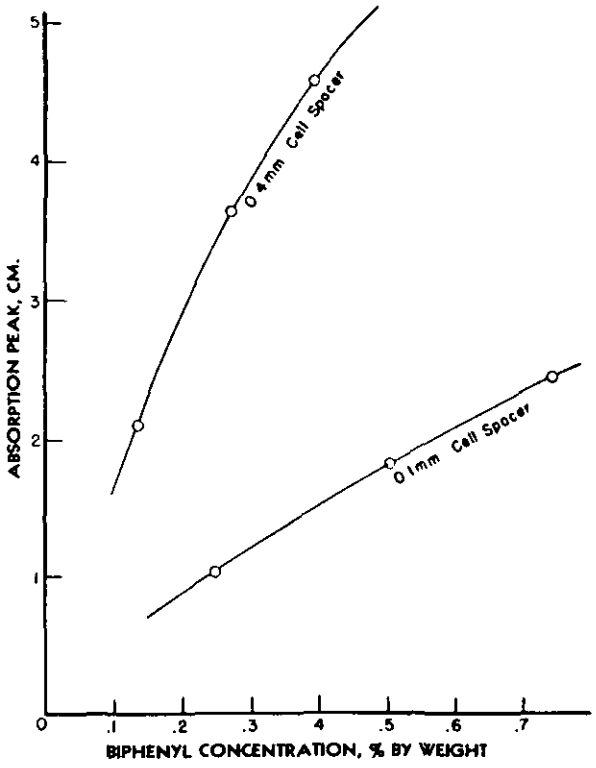

Figure 3. Standard Curves Obtained using 0.1- and 0.4-Mm. Spacers

In this modified isolation procedure, either peel or one or more whole fruit is ground in a Waring Blendor with water. The mash is transferred to a 3-liter round-bottomed flask and diluted with water to a volume of about 1.5 liters. During refluxing, orange oil distilled from the fruit acts as the extractant in a continuous liquid-liquid extractor (Figure 1), which is an oil separatory trap of the type used for the determination of volatile oils lighter than water (*1*). Juice samples (10 to 12 fruit) are analyzed in a similar manner, with the exception that for samples of juice obtained by reaming fruit (as contrasted with pressed juice) 1 ml. of *d*-limonene is added to the sample before refluxing. This addition is necessary because of the extremely low orange oil content of reamed juice samples. *d*-Limonene is used because it comprises about 95% of steam-distilled orange oil and its optical properties are identical with those of steam-distilled orange oil when the percentage biphenyl is calculated using a base-line technique (Figure 2).

The entire apparatus (Figure 1) is tilted as shown, so that during an analysis the oil layer remains well above the graduated measuring buret. If this is not done, orange oil will not separate properly in the small-bore buret tube and will flow back through the return tube into the round-bottomed flask. It has been found that no biphenyl vapor is lost if an Allihn type condenser is used. The drip tip of the condenser is removed so that the condensate flows down the walls of the separatory trap without agitating and emulsifying the oil layer. Serious losses of biphenyl were found to occur if the condensate dripped directly into and emulsified the orange oil phase.

After the sample of juice, peel, or whole fruit has been refluxed for 2 hours, the volume of oil is recorded and the oil sample is run into a small vial containing anhydrous sodium sulfate to remove traces of water. This oil sample is used to fill the infrared absorption cell.

The infrared absorption of the sample is recorded at high speed over the 13.90- to 14.60-micron range. Slit widths of 1.55 and 1.90 mm. are used for the 0.1- and 0.4-mm. spacers, respectively. For each analysis the gain is adjusted at 13.90 microns to give 65% transmittance with the sample in the light path. Each sample is recorded in duplicate and the average peak measurement used. However, if the variation between duplicates is more than 5% the analysis is repeated.

The absorption peak is measured from a base line drawn between the wave-length marker indentations at 14.16 and 14.58 microns (in Figure 2). A standard curve is prepared for each group of samples from analysis of standard solutions. These standards, once prepared, may be used continuously over a period of 1 to 2 months. Figure 3 shows typical standard curves in which the absorption peak, measured in centimeters, is plotted against percentage by weight of biphenyl.

RESULTS

Table I shows results obtained when known amounts of biphenyl were added to samples of whole fruit or samples of juice refluxed in the liquid-liquid extractor. Check runs were made by adding either solid biphenyl (samples 1 to 9) or 1 ml. of a standard solution of biphenyl in *d*-limonene (samples 10 to 31) to the sample. Samples 1 to 9 were analyzed using a 0.1-mm. cell spacer, whereas for all others a 0.4-mm. spacer was used.

DISCUSSION

The results shown in Table I indicate good recovery for the liquid-liquid type extractor. This method has the advantage, over the conventional steam distillation first used that analyses can be made on samples as small as a single fruit; moreover, entrainment is not a problem. Larger samples may be handled conveniently by employing a larger round-bottomed flask and a larger measuring buret in the oil separatory trap.

All results of the infrared analyses were calculated using the base-line technique in order to compensate for varying background absorptions of different samples of orange oil. Using this technique, standard solutions of biphenyl in *d*-limonene yield standard curves, such as the ones shown in Figure 3. With such

curves, considerable accuracy has been achieved in calculating the biphenyl content of solutions containing known amounts of biphenyl dissolved in a variety of orange oils.

The measurement of the absorption peak is arbitrary but convenient. It is done using a millimeter rule and measuring the vertical distance between the parallel lines shown in Figure 2. A new standard curve must be prepared for each group of samples because of variations in the machine's response with time. Using a 0.4-mm. spacer, analyses are accurate within the range of about 0.08 to 0.8% biphenyl. Samples more concentrated than 0.8% may be diluted with *d*-limonene.

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Quantitative Determination of Dissolved Oxygen in Nitrite-Containing Water Using Acid-Chromous Reagent

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IN A paper by Stone and Eichelberger (*5*) on the determination of dissolved oxygen in aqueous solution a method was developed for quantitatively determining dissolved oxygen by titration with a standard solution of acid-chromous reagent. However, this method did not give accurate results when nitrites were present in the water sample. The purpose of this research was to investigate a suggested modification of the original method, involving first the determination of the total number of equivalents of chromous solution necessary to titrate the nitrite and oxygen in an aliquot portion of the sample, and then the determination of the equivalents necessary to titrate the nitrite in another aliquot portion from which the oxygen had been removed by boiling. From these data the dissolved molecular oxygen could be determined by difference.

If this suggested modification proved unworkable, a method was to be investigated of determining the dissolved oxygen directly after first destroying the nitrites in the water sample and then determining the oxygen. This nitrite destruction method is essentially that suggested by Rideal and Stewart (*3*). It involves the use of permanganate to destroy the nitrite and then oxalic acid to destroy the excess of permanganate. This is followed by the use of the manganous sulfate, alkaline-iodide Winkler method (*6, 8, 9, 10*) of determining dissolved oxygen in the water sample.

EXPERIMENTAL

In the acid-chromous method employed by Stone and Eichelberger (*5*) carbon dioxide, free from oxygen, is run through the titration flask at a rate of approximately 400 ml. per minute for 3 minutes before the titration is started and at the same rate during the titration. An estimated excess of 0.02*M* chromium(II) reagent is run into the titration flask from the storage buret (*4*). The volume necessary to react with the oxygen of the sample and yield an excess is determined by a preliminary titration. A 5-ml. aliquot of the water sample is then added to the excess of chromium(II) reagent.

The excess chromium(II) reagent reacts with an excess of 0.0035*N* potassium iodate dispensed from a storage buret. A crystal of potassium iodide is added and the iodine equivalent to the excess iodate titrated with the chromium(II) reagent using starch as the indicator. The total equivalents of chromium(II) less the equivalents of iodate equal the number of equivalents of dissolved oxygen in the aliquot.

During the preliminary work, it was found that an end-point correction was necessary. This correction increased with an increase in the concentration of the starch in the reaction mixture. This concentration was necessarily high because of the small total volume of reactants. This factor and the many mechanical difficulties caused by the small size of the aliquot led to the introduction of a larger scale in the method. The volume of the sample was increased to 50 ml. and the concentration of the chromium(II) reagent was increased to 0.05*M* in chromium and in 0.1*M* hydrochloric acid.

Use of the iodate storage buret (*4*) was eliminated, and instead the iodate was dispensed from a plunger-type pipet with a known volume of less than 1 ml. The 0.0035*N* iodate solution was kept in an ordinary glass-stoppered bottle. This meant that the iodate solution was nearly saturated with dissolved oxygen. However, when the iodate was standardized against the chromium(II) reagent and used in the titration of a 50-ml. aliquot of water sample, the error introduced by fluctuations in the oxygen content of the iodate solution did not exceed 0.1%, which is less than the random error.

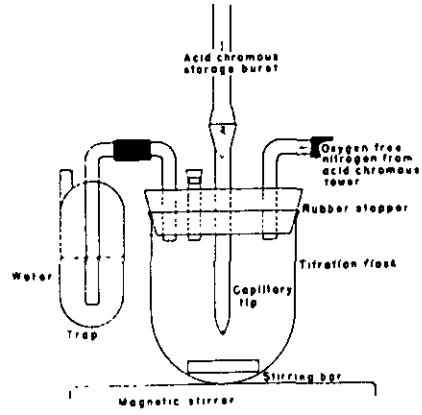

Figure 1. Diagram of Titration Flask

Trap on titration flask prevents diffusion of air into titration vessel. Tube next to capillary tip is used to introduce indicator reagent; it is stoppered at all other times

The grease used on the stopcocks of the chromium(II) reagent storage buret created a problem by "creeping" into the 2-ml. microburet bore. This difficulty was overcome by substituting Teflon-coated stopcock plugs with pressure adaptors.

REVISED PROCEDURE

Oxygen-free nitrogen is passed through the titration flask (Figure 1) at the rate of approximately 400 ml. per minute for 3 minutes before the titration is started, and at the same rate during the titration. After the 3-minute deaeration period, an estimated excess, determined by a preliminary titration, of 0.05*M* chromium(II) reagent is run into the flask. A 50-ml. aliquot

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A METHOD FOR THE DETERMINATION OF BIPHENYL IN ORANGES

By D. R. Beasecker

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A METHOD FOR THE DETERMINATION OF BIPHENYL IN ORANGES

An ultraviolet absorption analysis has been developed for the determination of biphenyl in oranges. The method is similar to the tentative procedure previously reported except for a few modifications. The procedure involves four steps; viz., (1) the isolation of biphenyl by steam distillation, (2) the extraction of the distillate with n-heptane, (3) the oxidation of orange oil in the extract with potassium permanganate, and (4) the measurement of biphenyl concentration in the extract. The first two steps have been combined through use of a liquid-liquid type extractor.

The method meets the following requirements of a "suitable method" as set up at the meeting held at the Institute of Paper Chemistry, April 6, 1953.

1. The method should be capable of measuring as little as as one part per million of biphenyl in orange peel with a limiting error of $\pm$ 0.1 part per million (ppm.) at the one part per million level.

2. The method should be absolute; i.e., no correction for orange oil interference should be required.

3. The method should be capable of analyzing as small a sample as one unit of fruit.

4. The elapsed time for an analysis should be a maximum of eight hours.

5. The method should be simple, so that it can be operated in a reasonably equipped laboratory with technically-trained personnel.

6. The method should be economical in terms of manpower, equipment, and reagents.

7. The method should be specific for biphenyl.

8. The method should be easily calibrated against known samples.

A ninth requirement which states that "the method should be applicable to other citrus fruits" has not been met. Preliminary studies with lemons show that interfering components are present which are not readily removed by the oxidation step.

Procedure

1. Peel the fruit with a pair of fla -tipped forceps, avoiding contact of hands with the peel as much as possible.

2. Weigh the peel within ± 0.1 gms. and the pulp within ± 1.0 gms. on a torsion balance. Treat the peel and pulp as separate samples.

3. Mince the peel (or pulp) in a ring blender for approximately three minutes using 500 . . of water as diluent for the peel and 100 ml. for the pulp.

4. Transfer the mixture to a 2000 ml., 24/40 standard taper, round bottom flask. Rinse blender thoroughly and dilute contents of flask to approximately 1000 ml. with water.

5. Connect flask to extraction system.

6. Add approximately 40 ml. of water and 60 ml. of n-heptane in that order to the extractor.

7. Connect extractor to an Allihn-type condenser. The water and heptane may be added through the top of the condenser if desired.

8. Extract for two hours. Regulate the reflux rate until condensation occurs in the middle bulb of the condenser. Under these conditions approximately 1500 ml. of condensate pass through the extractor. Most efficient extraction is obtained by allowing the condensate to run down the sides of the extractor rather than allowing it to drip directly into the heptane.

9. Allow the system to cool for 15 minutes.

10. Drain the extractor at the stopc into a 125 ml. separatory funnel.

11. Discard the water layer and drain heptane into a 100 ml. volumetric flask.

12. Rinse the condenser and extractor with 15 ml. of heptane by pouring through the top of the condenser. Drain the rinse liquid into the separatory funnel, swirl, and then drain into the volumetric flask.

13. Repeat step 12.

14. Cool contents of flask to room temperature and dilute to volume.

15. Add 50 ml. of three percent potassium permanganate solution (solvent-50% acetic acid) to a 125 ml. separatory funnel.

16. Add approximately 15 ml. of the heptane extract and stopper. A blank, using 15 ml. of heptane is prepared in the same manner.

17. Shake vigorously until the permanganate color disappears. The reaction is completed within three or four minutes. Release pressure in funnel occasionally.

18. Allow to set for a few minutes and then discard the lower layer.

19. Continue to add 50 ml. portions of permanganate solution in the above manner until the permanganate color persists. Three such treatments usually suffice for the peel extracts. One treatment is adequate for pulp extracts and the blank.

20. Add 15 ml. of glacial acetic acid and one milliliter of six percent potassium permanganate solution. Shake for approximately three minutes.

21. Add 75 ml. of water and shake vigorously for three minutes. Drain bottom layer.

22. Repeat steps 20 and 21 if permanganate color does not persist.

23. Add 50 ml. of two percent sodium carbonate solution, shake, and discard bottom layer.

24. Pipette 10 ml. of the peel extract into a 100 ml. volumetric flask and dilute to volume with heptane.

25. Measure the absorbancies of the diluted peel extract and the undiluted pulp extract. With the Cary Recording Spectrophotometer the following conditions were used:

    a. Wavelength - 248 mu.
    b. Slit - 0.52 mm.
    c. Dynode - position 4
    d. Cells - 10 mm. quartz
    e. Reference solvents -

        (1) For diluted extracts - the heptane used for dilution
        (2) For undiluted extracts - the heptane from blank sample

26. Using the absorbance determine the milligrams of biphenyl per 100 ml. of solution from the calibration curve.

27. Calculate the biphenyl content as follows:

$$\frac{\text{Reading from curve X dilution factor X 1000}}{\text{weight of sample}} = \text{ppm. of biphenyl in sample}$$

Sample calculation -

The extract of 140.1 gm. of peel diluted 10-100 gave an absorbance of 0.778 optical density units. This absorbance is equivalent to 7.10 mg. of biphenyl per 100 ml. of solution.

$$\frac{7.10 \text{ X } 100/10 \text{ X } 1000}{140.1} = 50.8 \text{ ppm. biphenyl in the peel}$$

28. If the biphenyl content of the peel is less than 5 ppm., repeat steps 20 through 27, omitting 24, until the biphenyl content is constant within ± 0.1 ppm. This additional treatment removes interfering traces of residual orange oil.

Calibration

Standard biphenyl solutions of the following concentrations were prepared from a 0.0100% solution of biphenyl in heptane: 2.50, 2.00, 1.50, 1.00, 0.50, and 0.20 mg./100 ml. of solution. The abosrbancy of each solution was measured using the conditions given in step 25. A plot of absorbancy (optical density) versus concentration of biphenyl showed perfect agreement with Beer's Law.

Apparatus

1. A liquid-liquid extractor similar to the design of attached drawing. Ace Glass Company sells a chloroform-water extractor which may be suitable. It is listed under Catalog No. 6881.

2. Allihn condenser, 300 mm. long and fitted with 24/40 standard taper joint at the bottom. Fisher Scientific Catalog No. 7-736.

3. Two-liter capacity, round bottom flasks with 24/40 standard taper joint.

4. Separatory funnels, 125 ml. volume, glass stoppered.

5. Volumetric flasks, 100 ml. volume, glass stoppered.

6. Cary Recording Spectrophotometer. A Beckman Model DU instrument would be satisfactory.

7. One pair of one centimeter quartz absorption cells.

8. Waring Blender, or equivalent.

Reagents

1. Normal heptane from Phillips Petroleum Company, pure grade, 99 mole percent minimum purity. The heptane is purified further by shaking vigorously in a separatory funnel with an equal volume of three percent potassium permanganate for five minutes, draining the bottom layer, and then washing with water and two percent sodium carbonate solution, respectively.

2. Six percent potassium permanganate solution. Prepared by dissolving 60 gms. of Baker and Adamson Reagent Grade permanganate in a liter of distilled water.

3. Three percent potassium permanganate solution in 50 percent acetic acid. Prepared by mixing equal quantities of six percent permanganate (2) and glacial acetic acid. This solution is not too stable and is prepared fresh every day.

4. Two percent sodium carbonate solution. Prepared by dissolving 20 gm. of Baker and Adamson Reagent Grade sodium carbonate in a liter of distilled water.

5. Biphenyl, Monsanto Chemical Company product.

Test Data

Four mixtures of orange oil and biphenyl were prepared and analyzed to determine the efficiency of the oxidation. The results are as follows:

Sample	% Oil	% Biphenyl Added	% Biphenyl Found	% Error
A	4	0.120	0.120	0.0
B	4	0.080	0.080	0.0
C	4	0.040	0.039	-2.5
D	4	0.020	0.019	-5.0

The absorption of Sample B, before and after treatment, was measured from 230 to 280 millimicrons. The spectra show the effectiveness of the oxidation. See attached spectra.

The efficiency of the method was tested by adding heptane solutions of biphenyl to samples of peel and pulp. Two untreated Valencia oranges were used for each sample. The biphenyl values reported in the table below have been calculated in terms of the amount of peel (or pulp) sample used.

Test	Type of Sample	Biphenyl Added (ppm.)	Biphenyl Found (ppm.)	% Error
1	Peel	1000	990	-1.0
2	Peel	500	480	-4.0
3	Peel	300	290	-3.3
4	Peel	100	97	-3.0
5	Peel	50.0	50.0	0.0
6	Peel	11.0	10.9	-0.9
7	Peel	1.73	1.90	+9.8
8	Peel	1.00	.90	-10.0
9	Pulp	1.06	1.10	+3.8
10	Pulp	0.41	0.39	-4.9
11	Pulp	0.20	0.19	-5.0
12	Pulp	0.20	0.18	-10.0

The efficiency of the extraction was tested by analyzing biphenyl treated Valencia oranges purchased from a grocer. The usual two hour extraction was made. The heptane extract and water were then drained from the extractor. The water was put back into the extractor and fresh heptane added. The extraction was continued for a second two hour period. Each heptane extract was analyzed for biphenyl. The peel of two oranges was used for each test. The pulps were also analyzed, but only one extraction was made. These tests show that the two hour extraction is adequate.

Test	Type Sample	Biphenyl from 1st Extraction	Biphenyl from 2nd Extraction	% Recovery of 1st Extraction
13	Peel	50.8 ppm.	0.93 ppm.	98.3
	Pulp	0.32 ppm.		
14	Peel	43.4 ppm.	1.48 ppm.	96.6
	Pulp	0.48 ppm.		

References

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2. Steyn, A. P., and Rosselet, F., Analyst, 74, 89 (1949).

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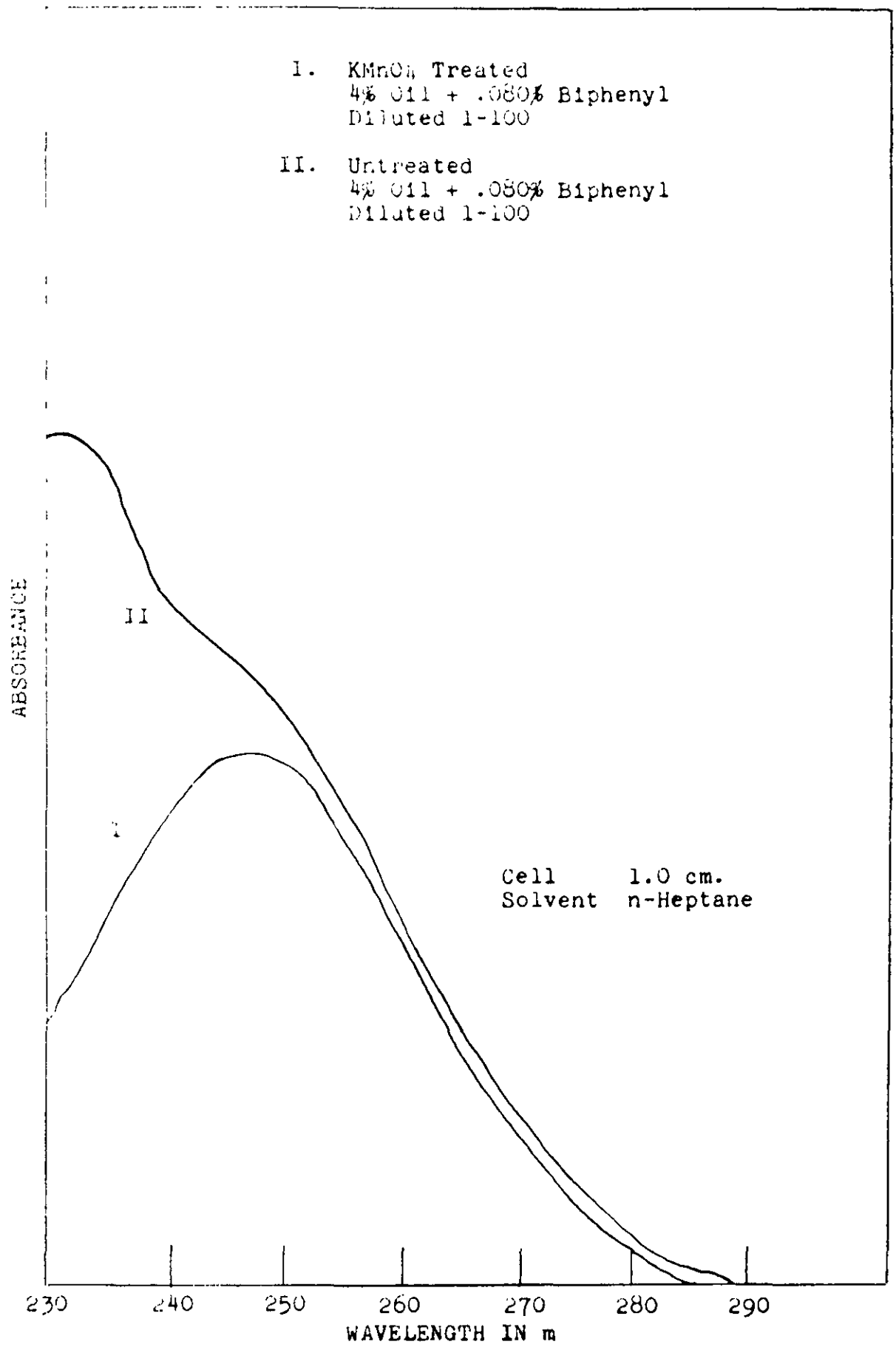
I. KMnO4 Treated
4% Oil + .080% Biphenyl
Diluted 1-100
II. Untreated
4% Oil + .080% Biphenyl
Diluted 1-100
ABSORBANCE
II
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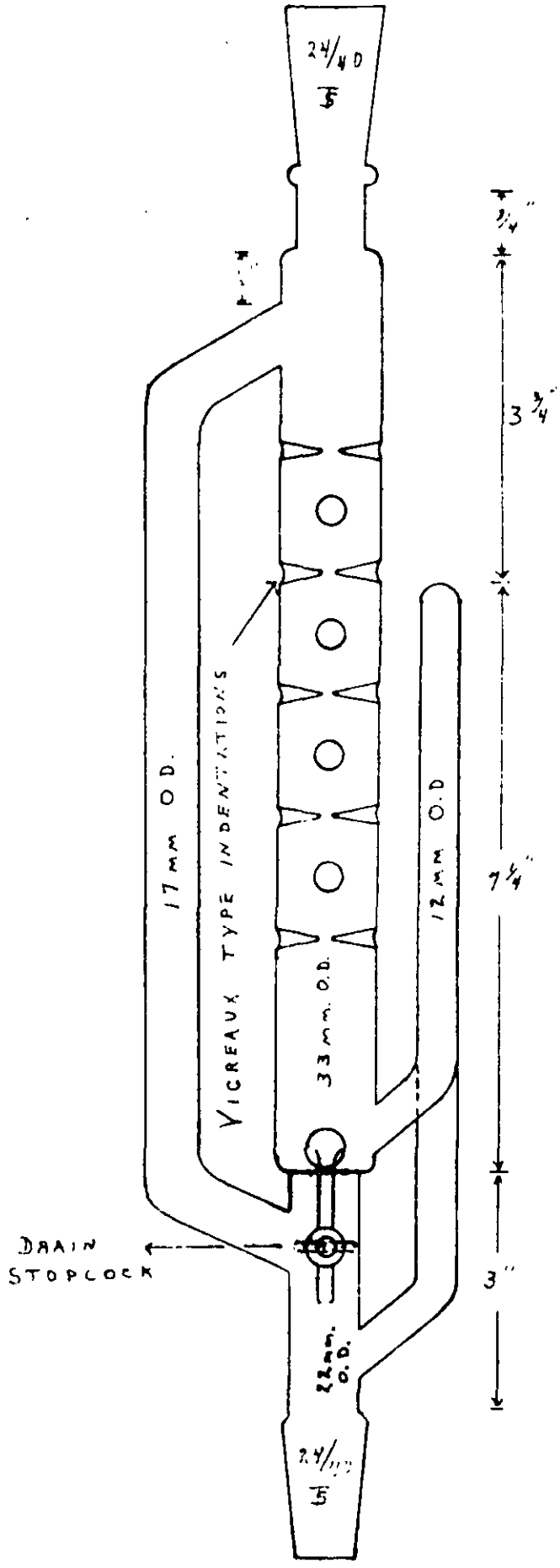
Cell 1.0 cm.
Solvent n-Heptane
230
240
250
260
270
280
290
WAVELENGTH IN m



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