A STUDY OF THE ASSAY OF PODOPHYLLUM.*

BY C. N. SPRANKLE AND C. B. JORDAN.

Podophyllum peltatum, commonly known as May Apple or Mandrake root has been used in medicine for ages, in fact, the Indians used it as an anthelmetic and emetic long before the discovery of this country (1). The crude drug and an extract of it were both included in the first U. S. P. of 1820. It was used as a cathartic. The resin, which was first discovered by Dr. John King in 1835 and reported in 1844 (2), was not included until the Fourth Revision of the U. S. P. It was called podophyllin.

The resin was first prepared by making a strong tincture of the drug, evaporating the tincture to the consistency of a thin syrup and adding it slowly, with constant stirring, to cold water, which precipitates the resin. The Fifth Revision required a saturated solution of alum as the precipitant, but this was not satisfactory as the resin was contaminated with the alum. The Sixth Revision required water acidulated with HCl and this precipitant has been used ever since.

R. Bentley in 1861 (3) found that this resin was made up of two resins; one soluble in alcohol and ether, the other soluble only in alcohol. The ether-soluble resin was the active portion; the ether-insoluble portion was inactive and called podophyllic acid. Buchheim in 1873 (4) reported that caustic alkalies converted the ether-soluble portion into an acid modification which was identical with the acid mentioned above. Therefore, he concluded that the active resin must be the anhydride of this acid.

F. B. Power in 1877 (5) verified the presence of two resins in podophyllin and he also found that podophyllin was not a true resin, since it was almost completely soluble upon prolonged treatment with hot water.

Podwissotzki in 1881 (6) published his outstanding investigations of the constituents of podophyllin. He isolated podophyllic acid by making a chloroform extract of the resin and precipitating the podophyllic acid in ether. He then added the ether-chloroform solution to petroleum ether and obtained a heavy, white noncrystalline precipitate of strong physiological action which he called podophyllotoxin. He treated the podophyllotoxin in alcohol with freshly slaked lime, evaporated to dryness and extracted with boiling alcohol, which, upon cooling, deposited long, snow-white, silky crystals which he stated to be active, but unsuitable for use because of its insolubility. He called it picropodophyllin (picro = bitter). He next treated podophyllotoxin with ammonia water and obtained an acid substance which he called picropodophyllic acid. He believed that podophyllotoxin was the product of reaction of picropodophyllin and picropodophyllic acid.

He also isolated a yellow coloring principle from the residue left after the chloroform extraction of podophyllin. This principle so resembled quercetin that he called it podophylloquercetin.

Kürsten in 1891 (7) stated that picropodophyllin is an isomer of podophyllotoxin and not a constituent of it as Podwissotzki had stated. He also stated that some picropodophyllic acid is formed by action of alkalies on podophyllotoxin, but not by hydrolysis.

---

* Scientific Section, A. PH. A., Toronto meeting, 1932.

Dunstan and Henry in 1898 (8) verified Podwissotzki's and Kürsten's work and, in addition, clarified the relationship of picropodophyllin and picropodophyllic acid to podophyllotoxin. They stated that podophyllotoxin heated with alkalies gave, by hydration, a salt of an unstable gelatinous acid which they termed podophyllic acid and which they also claimed to be the same as the picropodophyllic acid isolated by Podwissotzki and Kürsten. This acid readily loses a molecule of water to form the crystalline picropodophyllin which is isomeric with podophyllotoxin. It is also quite possible that all of the podophyllic acid does not lose a molecule of water and, hence, we have an explanation of Podwissotzki's supposition that podophyllotoxin yielded both picropodophyllic acid and picropodophyllin on hydrolysis. In addition to the above constituents, Dunstan and Henry isolated an amorphous resinous substance of strong purgative property from the residue left after removal of podophyllotoxin and podophylloquercetin. They called this substance podophylloresin.

It is quite evident that the official resin of podophyllum is not a single substance, but a mixture of complex substances. Podophyllotoxin and podophylloresin are both conceded to be physiologically active, but it has not been definitely proved that they alone are more effective than the combination of all the constituents.

EXPERIMENTAL.

(Podophyllum, podophyllin and podophyllotoxin were purchased from the Eimer & Amend Company.)

The podophyllotoxin purchased was noted to be of a dirty greyish yellow color rather than white as had been reported. Hence, its solubility in alcohol, chloroform, ether and water was tested and it was found to be only partially soluble in all except alcohol. This indicated an impure or chemically altered product, if reports in literature were correct. Podophyllotoxin was then prepared by extracting podophyllin with chloroform, evaporating to the consistency of a thin syrup, precipitating the podophyllic acid in ether and finally adding the ether-chloroform solution slowly to twenty times its volume of petroleum ether. A heavy, flocculent, white precipitate, resulted. This product was filtered, redissolved in chloroform, reprecipitated in petroleum ether, filtered, washed with petroleum ether and dried at room temperature. This final product was a fine, white crystalline substance which was soluble in alcohol, chloroform and ether, but only a part of it formed a colloidal solution in hot water.

Kremel in 1889 (9) and Eder and Schneiter in 1926 (10) proposed assays for content of podophyllotoxin in podophyllum and podophyllin by extracting with chloroform and precipitating in petroleum ether, but this is not satisfactory since it does not remove the podophyllic acid which is inert physiologically. Gordin and Merrill in 1903 (11) proposed an assay for content of podophyllotoxin in podophyllin by treating the podophyllin with freshly slaked lime in the presence of alcohol to convert the podophyllotoxin to picropodophyllin, filtering, evaporating an aliquot part to dryness and weighing it as picropodophyllin.

This quantitative conversion of podophyllotoxin into its isomer, picropodophyllin, was attempted but without success.

Since podophyllotoxin is not the only active constituent, it was decided to return to the resin itself.

There was no color test found in literature for podophyllin. In an attempt to find a color test to be used in determining complete extraction of the resin from the drug, all of the common alkaloidal, protein and qualitative reagents were added to either an ammonia water or glacial acetic acid solution of the resin, but none proved satisfactory.

However, there was one which gave indications of being of value. To an ammoniacal solution of the resin, a 5% solution of $CuSO_4$ was slowly added. A dark green color resulted instead of the expected characteristic deep blue color of the copper-ammonia complex ion. This green color darkened somewhat and persisted until a large excess of copper sulphate had been added which then gave the characteristic deep blue color. But it was found that an ammonia water extract of the marc left after complete extraction of the resin gave the same color as also did other resins, for example: Jalap, scammony, etc.

Podophyllin was prepared by the U. S. P. method, substituting different solvents for alcohol with the following results:

TABLE I.

Solvent.	Total Extractive.	Per Cent Resin.
Alcohol	....	3.65
Acetone	....	3.54
Alcohol and acetone (1 + 1)	....	3.63
Alcohol and ether (1 + 1)	....	3.02
Alcohol (after maceration with excess of water)	11.374	3.59
Acetone (after maceration with excess of water)	11.395	3.60

Acetone is a much more rapid solvent than alcohol, but a larger amount is required for complete extraction. Too much water was used in the last two extractions and the drug swelled to almost twice its normal volume. Percolation was very, very slow and, upon evaporation, a very disagreeable mass resulted from which it was difficult to remove the resin.

Alcohol seems to be the best and most practical solvent.

QUANTITATIVE ANALYSIS OF THE DRUG.

Because of the complexity of this resin, it was decided to assay the drug for total content of resin using alcohol as the solvent. The assay methods applied to the drug were: U. S. P. IX, U. S. P. X, Jenkins' (12) and Warren's modification of Jenkins' method (13).

The following is a brief outline of the four methods listed:

The U. S. P. IX Method.—Make a strong tincture by cold maceration and percolation of a ten-Gm. sample, evaporate to the consistency of a thin syrup and add slowly with constant stirring to 10 cc. of 0.6% HCl water. Filter and dry the resin to constant weight at 80° C.

The Jenkins' Method.—Digest a 10-Gm. sample on a water-bath for three hours with 25 cc. of alcohol, transfer to a percolator, percolate slowly till 45 cc. are collected. Cool and make up to 50 cc. Mix thoroughly. Transfer a 10-cc. aliquot part to a separator, add 10 cc. of 0.6% HCl and 10 cc. of chloroform and shake. Draw off the alcohol-chloroform layer into another separator, wash the acid layer twice with 15-cc. portions of alcohol-chloroform mixture (1 + 2). Mix the washings with the extraction in the second separator, add 10 cc. of 0.6% HCl, shake and draw off the lower layer into a tared vessel. Wash the acid layer twice as before with the

alcohol-chloroform mixture, adding the washings to the tared vessel. Evaporate to dryness and dry to constant weight at 100° C.

The U. S. P. X Method.—Digest a 10-Gm. sample with 60 cc. of alcohol for three hours as above and collect 95 cc. of percolate. Cool, make up to 100 cc. and transfer 20-cc. aliquot part to a separator. Add 20 cc. of a saturated solution of potassium citrate (20 Gm. of potassium citrate in 12 cc. of water) and 10 cc. of chloroform. Shake well and allow to separate over night. Draw off potassium citrate layer and discard. Then draw off alcohol-chloroform layer, filter it into a tared vessel and rinse out the separator with alcohol-chloroform mixture (2 + 1). Evaporate to dryness and dry to constant weight at 100° C.

L. E. Warren's Modified Jenkins' Method.—Prepare the tincture as by the U. S. P. X method except for one-half hour digestion. Take 10-cc. aliquot part in a separator and proceed as in the Jenkins' method, using 0.6% HCl as the precipitant, but wash the acid layer three times instead of twice with the alcohol-chloroform mixture. Evaporate the final extractions just to dryness, add 1 cc. of dehydrated alcohol, again evaporate to dryness and dry to constant weight at 80° C.

The results were:

TABLE II.

U. S. P. IX.	U. S. P. X.	Jerkins'.	Warren's Modification.
3.86%	6.58%	6.91%	6.60%
3.88%	6.82%	7.07%	6.68%
3.72%	27.90%	5.90%	6.71%
3.60%	14.79%	5.43%	6.53%
	9.94%	8.08%	
	17.16%	7.38%	
		6.82%	
		6.98%	

The U. S. P. IX method is a cold percolation method, whereas the others are hot digestion and percolation methods. It will be noted that the hot processes yield a much higher per cent of resin than the cold percolation method. The marc from two of the U. S. P. IX samples were subjected to the Jenkins' method and gave additional resin as follows: 0.85%, 1.15%. This additional resin conformed to the U. S. P. requirements for the resin. The U. S. P. IX process is long and more cumbersome than the others. The U. S. P. X method gave poor results because of contamination of the resin with potassium citrate. A bad feature of this assay is the formation of a thick, brown scum at the junction of the saturated potassium citrate solution and the alcohol-chloroform solution in the separatory funnel upon standing over night. In the first series this scum was drawn off and discarded with the saturated potassium citrate solution and the results checked closely, but the resin was contaminated with potassium citrate. In later series this scum was left with the alcohol-chloroform solution and filtered with it, but the results were very high. When the saturated solution of potassium citrate is drawn off, much of it clings to the stem of the funnel and is washed off on to a filter paper when the alcohol-chloroform solution is drawn off. This is undoubtedly a source of much of the contamination.

The scum from another series was drawn off and examined. It was found to be a super-saturated solution of potassium citrate with a little incorporated resin.

In addition to the above sources of error, some of the resin remains in the filter paper when the alcohol-chloroform solution is filtered. It is not washed through even by an excess of the alcohol-chloroform solvent.

The results by Jenkins' method did not check very closely. Quite likely this

is due to incomplete washing out of the resin in percolation, as only fifty cubic centimeters of percolate are collected.

The results by Warren's modification were very good. The only questionable point was the half-hour digestion. To determine whether length of time of digestion effects the results, four samples were run by the Warren method, except that they were digested three hours. The results were:

TABLE III.

Half-Hour Digestion.	Three-Hour Digestion.
6.71%	6.82%
6.53%	6.69%
6.60%	6.76%
6.68%	6.84%

An increase of one-tenth of one per cent resulted from this longer digestion.

The Jenkins' method was again applied to ten-gram samples, taking the precaution to percolate slowly by adding the alcohol in small portions to insure complete washing out of the resin. Jenkins' method directs washing the acidulated water in the separator twice with the alcohol-chloroform mixture. The above samples were washed three times and resulted in an increase as the following results will show.

TABLE IV.

Washing Two Times.	Washing Three Times.
6.74%	
6.45%	6.81%
6.63%	6.55%
6.58%	

Dr. W. L. Scoville of Parke, Davis and Company, suggested that the saturated solution of potassium citrate be made just acidic with acetic acid to prevent the scum formation. He also suggested that the alcohol-chloroform mixture be drawn off at the top of the separator after the saturated potassium citrate solution had been drawn off at the bottom. His first suggestion prevented the scum formation, but the latter suggestion did not prevent the contamination of the resin, for, after shaking the two solutions together in the separator, and allowing them to stand over night, some of the potassium citrate crystallizes out around the top and is washed on to the filter paper when the alcohol-chloroform solution is drawn off. The results obtained by following Dr. Scoville's suggestion were:

TABLE V.

	I.	II.	III.
Sample 1	12.71%	18.48%	9.01%
Sample 2	7.23%	12.16%	12.08%

The disadvantage of the U. S. P. X method is the contamination of the resin with potassium citrate. A possible error in the Jenkins' and Warren's methods is that some of the resin may be soluble in the weakly acid solution used for precipitation. To investigate this possible error, two samples of purchased podophyllin were dissolved in ten cubic centimeters of alcohol, transferred to a separator and subjected to the Warren method. A slight loss occurred as follows:

TABLE VI.

	I.	II.
Weight of sample	0.0734	0.1359
Weight recovered	0.0707	0.1331
Loss in weight	0.0027	0.0028

This is not truly indicative of positive loss, however, since this was a purchased sample and may have contained water-soluble material.

The Warren modification directs drying at 80° C. and the U. S. P. X and Jenkins' methods at 100° C. The higher temperature results in a much darker colored resin. When the solubility of these two products in alcohol were examined, it was found that the resin dried at 80° C. is completely soluble, whereas the resin dried at 100° C. is not completely soluble. No doubt chemical alteration takes place when the resin is dried at the higher temperature.

SUMMARY.

The U. S. P. IX method is long and does not extract all of the resin. The U. S. P. X method is not practical because of the potassium citrate which contaminates the final product. The Jenkins' method, if care is taken to percolate slowly by adding just a little alcohol at a time and if the acidulated water is washed three times with the alcohol-chloroform mixture, will give good results. The Warren modification of the Jenkins' method, with our suggested additional modification of digesting three hours instead of one-half hour, will give the best results and seems to be the most practical for inexperienced as well as experienced analysts.

If the cold process for the manufacture of the resin remains official in the U. S. P. XI, then the U. S. P. IX assay method would be the logical one for content of available resin. However, the assay should be for total content of resin. Should the revision committee wish to include an assay method which assays approximately twice the amount of resin that can be obtained by the official process of manufacture, then the following method is recommended to insure accurate results by the average analyst.

(The Jenkins' method, modified by L. E. Warren and further modified as suggested by us):

Place 10 Gm. of drug (No. 60 powder) in a 125-cc. Erlenmeyer flask and add 60 cc. of alcohol. Fit a stopper with a glass tube for refluxing (a reflux condenser may be substituted) and heat on a water-bath for three hours. Transfer to a small percolator and percolate slowly with warm alcohol till 95 cc. have been collected. Cool, make up to exactly 100 cc. and mix thoroughly.

Transfer 10 cc. of the above tincture to a separator, add 10 cc. of chloroform and 10 cc. of 0.6% hydrochloric acid. Shake, allow to separate and wash the acid layer three times with successive 15-cc. portions of alcohol-chloroform mixture (one volume alcohol and two volumes chloroform), adding the washings to the second separator. Add 10 cc. of 0.6% hydrochloric acid to the combined extract and washings, shake, allow to separate and draw off the alcohol-chloroform layer into a tared vessel. Wash the acid layer three times with 15-cc. portions of the alcohol-chloroform mixture, adding the washings to the tared vessel. Evaporate the combined extractions on a water-bath to apparent dryness, add 1 cc. of dehydrated

alcohol and again evaporate to dryness. Dry to constant weight at 80° C. Weight of residue multiplied by 100 gives the per cent of resin in the drug.

BIBLIOGRAPHY.

(1) Bennet, *Pharm. J.*, 70 (1903), 238.
(2) Lloyd, PROC. A. PH. A., 38 (1890), 357.
(3) Bentley, *Pharm. J.*, 21 (1861), 457.
(4) Buchheim, PROC. A. PH. A., 22 (1874), 125.
(5) Power, *Ibid.*, 25 (1877), 420.
(6) Podwissotzki, *Pharm. J.*, 41 (1881), 217, 1011.
(7) Kürsten, PROC. A. PH. A., 40 (1892), 575.
(8) Dunstan and Henry, *Pharm. J.*, 60 (1898), 213.
(9) Kremel, PROC. A. PH. A., 37 (1889), 398.
(10) Eder and Schneiter, YEAR BOOK, A. PH. A., 15 (1926), 391.
(11) Gordin and Merrill, PROC. A. PH. A., 50 (1902), 343.
(12) Jenkins, Jr., *Ind. & Eng. Chem.*, 6 (1914), 671.
(13) Warren, *J. A. O. A. C.*, 13 (1930), 117.

PERMANENT COLOR STANDARDS FOR U. S. P. COD LIVER OIL, ALMOND OIL AND CASTOR OIL.*

BY ABRAHAM TAUB.

The need for the more definite specification of color for certain U. S. P. oils has been frequently pointed out both by manufacturers and food and drug enforcement officials. To describe an oil as pale yellow in the U. S. P. monograph may lead to several ambiguous interpretations. It may imply that a colorless oil would not meet the U. S. P. requirements; or it may leave the observer in doubt as to whether the oil in question is slightly darker than pale yellow. Furthermore, the term pale yellow does not distinguish between greenish yellow and reddish yellow. Only a quantitative standard can obviate this condition.

While there are many instruments on the market for the specification of color, it is felt that a simple and inexpensive method would be more desirable for most laboratories. It is the purpose of this paper to set up permanent color standards for these oils by using the Arny "Co–Fe–Cu" inorganic colored fluids. The colors exhibited by the U. S. P. oils which were studied, required the use of only two of these inorganic fluids to produce proper matches—the $M/4$ $CoCl_2 \cdot 6H_2O$ and the $M/6$ $FeCl_3 \cdot 6H_2O$, both acidulated with 1% HCl to prevent hydrolysis and insure permanent stability.

For checking the closeness of the match, a Bausch & Lomb spectrophotometer was used. A number of the "Co–Fe" blends were made up which closely approximated the color of the oil being studied, and the particular blend whose spectrophotometric curve most nearly coincided with the spectrophotometric curve of the oil was adopted. The reason for using this instrument rather than a colorimeter or tintometer was to produce a match which would not appear different under different light conditions. It has been definitely shown that two liquids whose spectral transmission curves are alike will appear the same under different conditions of light; this is not necessarily true for two liquids which give the

* Scientific Section, A. PH. A., Toronto meeting, 1932.