# Analysis of the Alkaloid Content of Ergot

By THOMAS G. ALEXANDER and DANIEL BANES

A new procedure for the assay of ergot is presented. Total alkaloids are extracted with ammonia-methanol-chloroform and water-soluble alkaloids are separated by column chromatography. Total and water-soluble ergot alkaloid contents are determined colorimetrically. The relative concentrations of ergonovine and ergonovinine in the water-soluble alkaloid fraction are estimated by paper chromatography. The new procedure yields higher values for water-soluble alkaloid content than does the current N. F. procedure.

THE NATIONAL FORMULARY method (1) for the determination of the alkaloid content of ergot has several drawbacks. Extraction of total alkaloids from the ergot is sometimes incomplete under the conditions prescribed, and emulsions are often formed during the extraction, making the procedure tedious and time-consuming. In a collaborative study on a modified version of the N. F. procedure, Strong and Maurina (2) found a large variation in results reported from different laboratories for the alkaloid content of ergot samples. This study was undertaken in an effort to reduce variability in assay, and to permit a more accurate evaluation of ergot samples with respect to their ergonovine content.

Various methods for extracting alkaloids from ergot were investigated (3, 4, 5). The method proposed here involves a simple extraction of total alkaloids by shaking ergot with two immiscible solvents. Water-soluble alkaloids are then separated from water-insoluble alkaloids by a column chromatographic procedure based upon the findings of Levine (6) and Banes (7). Relative concentrations of ergonovine and ergonovinine in the water-soluble alkaloids fraction are estimated by means of a paper chromatographic procedure adapted from that described by Levine and Fischbach (8).

The classical chromogenic reagent, *p*-dimethylaminobenzaldehyde (9), has been employed for the colorimetric assay of ergot alkaloids since its use was first proposed by Smith (10). Burn (11) has suggested that the temperature be controlled carefully during addition of the *p*-dimethylaminobenzaldehyde solution in the determination of ergot alkaloids. When two volumes of *p*-dimethylaminobenzaldehyde T. S. are mixed with one volume of an aqueous solution of an ergot alkaloid, the temperature rises rapidly, and the intensity of the blue color developed in replicate tests is variable. If the flask is swirled continually during slow dropwise addition of the reagent to the alkaloidal solution, the color intensity may be increased by more than ten per cent. Reproducibility is improved and maximum colors are developed when the flask is cooled in an ice bath during dropwise addition of the reagent. (This was especially true in the presence of materials which may char, such as sugars.) These precautions were observed in devising the proposed method.

Received July 11, 1960, from the Department of Health, Education, and Welfare, Food and Drug Administration, Bureau of Biological and Physical Sciences, Division of Pharmaceutical Chemistry, Washington 25, D. C.

Accepted for publication August 1, 1960.

The authors are indebted to Sandoz, Inc. for supplying samples of ergot and ergot alkaloids, and to S. B. Penick and Co. and Eli Lilly and Co. for samples of ergot.

## ASSAY PROCEDURE

The whole procedure, including paper chromatography but excluding the assay of total alkaloids, must be carried out on the same day and in subdued light.

**Extracting Solvent.**—Dissolve 10 ml. of ammonium hydroxide, reagent grade, in 90 ml. of methyl alcohol and mix with 900 ml. of chloroform.

**Color Developing Reagent.**—U. S. P. XVI *p*-dimethylaminobenzaldehyde T. S. (12).

**Standard Solution.**—Weigh 6.78 mg. of ergonovine maleate U. S. P., dissolve in 1% tartaric acid solution, and dilute to 250 ml. with water. This solution contains the equivalent of 20 mcg. ergonovine per ml.

**Diatomaceous Silica Support.**—Boil 150 Gm. chromatographic siliceous earth[1] with 1 L. hydrochloric acid (1 + 1) for ten minutes and cool. Then wash with water to remove acid and dry in oven at 100°. The washed product should give no test for acid when moistened.

**Chromatographic Tube.**—Prepare chromatographic tube from 25 × 200-mm. test tube. Fit it with a packing rod about 400 mm. long, having flat head 22–23 mm. in diameter. Place a small wad of glass wool in the bottom of the tube.

**Extraction of Alkaloids.**—Weigh 5 Gm. of ergot, ground to No. 60 powder, into a 125-ml. separator. Add 50 ml. of extracting solvent and shake vigorously for five minutes. Add 50 ml. of chloroform and 2 ml. of water, shake gently, and allow to settle. Draw off the lower phase as completely as possible, and filter through a plug of glass wool into a 200-ml. volumetric flask. Add another 20 ml. of extracting solvent to the ergot residues in the separator, mix, add 20 ml. of chloroform and shake. Filter the lower phase through the glass wool into the volumetric flask. Repeat rinse procedure once with an

[1] Celite No. 545, Johns-Manville Co.

additional 20 ml. of extracting solvent and 20 ml. of chloroform. Dilute the combined extracts to 200 ml. with chloroform and mix.

**Preparation of Total Alkaloid Solution.**—Evaporate a 25-ml. aliquot of the chloroform-ammonia extract solution to dryness at a temperature not exceeding 40°. (If desired, dried sample may be stored overnight in a refrigerator for continuation of assay.) Dissolve the residue in 20 ml. of ether and transfer to a 125-ml. separator with the aid of three 20-ml. portions of ether followed by 5-ml. and 3-ml. portions of approximately 0.2 *N* sulfuric acid. (To check for complete transfer, add 2 ml. of acid to flask and examine under ultraviolet light. There should be no fluorescence.) Shake and draw off the acid layer into a 25-ml. volumetric flask through a plug of glass wool. Extract the ether layer with 5-, 5-, and 3-ml. portions of 0.2 *N* sulfuric acid. Combine the acid extract and washings and dilute to 25 ml. This solution is the *Assay Preparation of Total Alkaloids.*

**Preparation of Water-Soluble Alkaloid Solution.**—Place 5 Gm. of chromatographic siliceous earth and 5 ml. of 0.1 *M* citric acid in a beaker. Mix thoroughly until mixture appears fluffy and uniform, and transfer to the chromatographic tube. Tap side of tube gently to let mixture settle. Press down firmly and evenly with packing rod. Then mix 2 Gm. siliceous earth with 2 ml. of water and pack the mixture on top of the acid trap. Finally place a layer of glass wool on top of the column.

Evaporate a 150-ml. aliquot of the chloroform-ammonia extract to dryness at a temperature not exceeding 40°. There should be no odor of ammonia or of solvent. Dissolve the residue in 10 ml. of chloroform and transfer quantitatively to the acid trap column with the aid of several small portions of chloroform. Wash down the sides of the chromatographic tube with chloroform and then elute water-insoluble alkaloids with 125 ml. of water-saturated chloroform. Inspect for proper retention of water-soluble alkaloids by very briefly holding the column under an ultraviolet lamp. A brightly fluorescent blue ring in the citric acid layer indicates that the ergonovine has been properly retained. Discard eluent and glass wool cover. Extrude column containing sample with very gentle air pressure into a 400-ml. beaker, mix with 5 ml. of 10% sodium bicarbonate, then mix with enough siliceous earth to make a workable mixture and transfer quantitatively to the chromatographic tube. Rinse packing rod and stirrer with a small amount of chloroform into a beaker. Add enough siliceous earth to adsorb the chloroform and transfer to the column. Rinse again with chloroform. Pass 75 ml. of water-saturated chloroform through the column receiving the eluent in a 125-ml. separator containing 5 ml. of approximately 0.2 *N* sulfuric acid. Examine under an ultraviolet lamp to check for complete removal of water-soluble alkaloids from the column. There should be no fluorescence due to ergot alkaloids.

Shake the mixture of acid and eluate, and draw off the chloroform layer into a second separator. (If after standing fifteen minutes, the organic phase does not completely settle out free of emulsion, add about 30 mg. of anhydrous sodium sulfate and shake until it dissolves.) Transfer the acid layer through the mouth of the separator into a 25-ml. volumetric flask. Rinse first separator and then chloroform with 5-ml. portion of approximately 0.2 *N* sulfuric acid and add the rinse to the acid solution in the volumetric flask. Repeat rinse twice. Dilute the combined acid extracts to 25 ml. and mix. This is the *Assay Preparation of Water-Soluble Alkaloids.*

**Development of Color.**—Transfer 5.0-ml. aliquots of *Assay Preparations* and of *Standard Solution* to separate stoppered Erlenmeyer flasks. Immerse in an ice bath and add, dropwise, 10.0 ml. of Color Developing Reagent with constant swirling. Allow to stand at room temperature for forty-five to sixty minutes with occasional mixing. Determine the absorbance in a suitable spectrophotometer, at 550 mμ, using as reference blank a mixture prepared by adding 10.0 ml. of reagent to 5.0 ml. of 0.2 *N* sulfuric acid.

**Calculations.**—Total alkaloid content as per cent ergotoxine = $0.150 \times A/S$, where $A$ is the absorbance of the aliquot of *Assay Preparation for Total Alkaloids* and $S$ is absorbance of the Standard. Water-soluble alkaloid content, as per cent ergonovine = $0.01333 \times A/S$, where $A$ is the absorbance of the aliquot of *Assay Preparation of Water-Soluble Alkaloids* and $S$ is the absorbance of the Standard.

## PAPER CHROMATOGRAPHIC PROCEDURE

**Developing Solvent.**—Mix 100 ml. *n*-butyl acetate, 50 ml. nitromethane, 50 ml. ethylene glycol monoethyl ether acetate (Cellosolve acetate), 8 ml. pyridine, and 10 ml. water. Discard aqueous phase.

**Immobile Solvent.**—Prepare pH 6 sodium hydroxide-potassium phosphate buffer (13).

**Standard Solution.**—Dissolve 6.78 mg. ergonovine maleate U. S. P. in 2 ml. of alcohol. This solution contains the equivalent of 2.5 mcg. ergonovine per μL.

**Chromatographic Tanks.**—Assembly apparatus for ascending paper chromatography with 8 × 8 inch sheets.[2] Blotter paper liners must be used. Ascending paper chromatographic techniques and apparatus have been described in detail by Mitchell (14).

**Preparation of Sample.**—Render alkaline with sodium bicarbonate a 20-ml. portion of *Assay Preparation of Water-Soluble Alkaloids*, and extract alkaloids with four 10-ml. portions of chloroform. Evaporate combined extracts to dryness at a temperature not exceeding 40°. Just before spotting dissolve residue in 0.5 ml. of alcohol.

**Procedure.**—Equilibrate developing solvent in tank for thirty minutes. Pour onto marked 8 × 8 inch paper about 10 ml. of buffer solution. Blot with paper towels to remove excess moisture. Quickly spot 1, 2, and 5 μL. of standard solution and 5 and 10 μL. of sample solution. Moisten spots with water from a capillary tube. A spot at edge of paper of methyl red indicator in alcohol will aid in locating solvent front. When paper feels moist and limp, but not so wet as to transfer water to the fingers, place in tank. When solvent front is within an inch of the top of the paper, remove and dry in hood. Examine under ultraviolet lamp.

[2] Arthur H. Thomas Co., No. 3677, has been found suitable.

TABLE I.—COMPARISON OF ASSAY METHODS[a]

| Method of Assay | % Water-Soluble Alkaloids — Sample No. 2 | Sample No. 5 | Sample No. 6 | Sample No. 9 |
|---|---|---|---|---|
| N. F. percolation and separation | 0.0084 | 0.0100 | 0.0142 | 0.0218 |
| N. F. percolation column separation | 0.0087 | 0.0081 | 0.0114 | 0.0216 |
| 2-Phase extraction N. F. separation | 0.0123 | 0.0125 | 0.0181 | 0.0256 |
| 2-Phase extraction column separation | 0.0123 | 0.0124 | 0.0171 | 0.0279 |

[a] All assays by the same analyst.

Blue fluorescent spots of ergonovine appear about one-third to one-half the distance from starting line to the solvent front with ergonovinine spots immediately ahead.[3] The water-soluble alkaloids are equally fluorescent, thus a rough estimation of ergonovine and ergonovinine contents can be made by comparing the intensity of fluorescence of various sample spots with those of the standard spots.

## RESULTS AND DISCUSSION

The ammonia-methanol-chloroform combination proved the most effective of the extracting solvents compared. Some ergot residues from the N. F. method of percolation were extracted a second time by shaking. As much as 5% of the total alkaloids were extracted from the residues. In the case of the two-phase shaking method no more than 0.5% of total alkaloids or 1% of the water-soluble alkaloids were ever recovered in a second extraction. The variation in collaborative results reported by Strong and Maurina (2) may be due in part to the inefficiency of the single-phase extraction process. The inadequacy of the percolation method is further indicated in Table I which compares the two methods of extraction. Table I also shows that the N. F. and chromatographic column methods for the isolation of water-soluble alkaloids yield similar results.

The findings recently reported by Vining and Tabor (15) corroborate our conclusion that a two-phase system is necessary for extraction. These authors report that ergot alkaloids were best extracted from lyophilized saprophytic cultures of *Claviceps purpurea* with a small amount (1 ml.) of 10% ammonia solution and ether (50 ml.) or chloroform. They state that "wetting of the culture was necessary to release the alkaloid from adsorptive binding to the culture solids since none could be extracted with dry nonpolar solvents."

The 0.1 *M* citric acid trap was found to retain ergonovine and ergonovinine quantitatively. Ergotamine was quantitatively eluted from the citric acid trap with 70 ml. of chloroform and ergotoxine with 60 ml. To check recoveries of ergonovine, aliquots of a solution of ergonovine maleate U. S. P.

TABLE II.—COMPARISON OF RESULTS OBTAINED BY SEVERAL ANALYSTS[a]

| Analyst | Sample No. 3 — % Total Alkaloids | % Water-Soluble Alkaloids | Sample No. 7 — % Total Alkaloids | % Water-Soluble Alkaloids |
|---|---|---|---|---|
| 1 | 0.188 | 0.0160 | 0.201 | 0.0204 |
| | 0.188 | 0.0162 | 0.206 | 0.0208 |
| | 0.195 | 0.0163 | ... | 0.0208 |
| | 0.195 | 0.0163 | ... | 0.0208 |
| | ... | 0.0164 | ... | 0.0211 |
| | ... | 0.0167 | ... | 0.0213 |
| | ... | 0.0170 | ... | ... |
| 2 | 0.180 | 0.0159 | 0.193 | 0.0204 |
| | 0.181 | 0.0163 | ... | ... |
| 3 | 0.187 | 0.0161 | ... | ... |
| | 0.188 | 0.0161 | ... | ... |
| 4 | 0.176 | 0.0163 | 0.185 | 0.0209 |
| | 0.176 | 0.0164 | 0.186 | 0.0210 |
| Average | 0.185 | 0.0163 | 0.194 | 0.0208 |

[a] Each pair of figures represents a separately weighed and analyzed portion of ergot. The authors are indebted to the following for their help in this collaborative study: J. Wolff, Div. of Pharm. Chem., Food and Drug Admin.; S. Cohen, Minneapolis District, Food and Drug Admin.; J. Salas, Caracas, Venezuela.

[3] If the spots diffused badly, it indicates that paper was too wet when placed in tank. If spots did not move, paper probably was too dry.

TABLE III.—COMPARISON OF RESULTS OBTAINED USING N. F. AND PROPOSED PROCEDURES[a]

| Sample Number | N. F. Method — % Total Alkaloids | % Water-Soluble Alkaloids | Proposed Method — % Total Alkaloids | % Water-Soluble Alkaloids |
|---|---|---|---|---|
| 1 | 0.201 | 0.0197 | 0.224 | 0.0264 |
| | 0.189 | 0.0198 | 0.228 | 0.0262 |
| 2 | 0.156 | 0.0081 | 0.163 | 0.0117 |
| | 0.160 | 0.0084 | 0.166 | 0.0136 |
| | | | 0.158 | 0.0126 |
| | | | 0.177 | 0.0125 |
| | | | 0.182 | 0.0124 |
| 3 | 0.204 | 0.0160 | 0.188 | 0.0163 |
| | 0.209 | 0.0166 | 0.188 | 0.0163 |
| | 0.207 | 0.0166 | 0.195 | 0.0170 |
| | 0.202 | 0.0152 | 0.195 | 0.0164 |
| | 0.191 | 0.0158 | ... | 0.0167 |
| | | | ... | 0.0160 |
| | | | ... | 0.0162 |
| 4 | 0.148 | 0.0089 | 0.178 | 0.0119 |
| | .146 | 0.0092 | 0.158 | 0.0137 |
| | .140 | 0.0099 | 0.163 | 0.0136 |
| | | | 0.162 | 0.0137 |
| 5 | 0.129 | 0.0100 | 0.150 | 0.0121 |
| | | | 0.140 | 0.0127 |
| 6 | 0.161 | 0.0142 | 0.190 | 0.0169 |
| | | | 0.185 | 0.0173 |
| 7 | 0.210 | 0.0231 | 0.201 | 0.0213 |
| | 0.212 | 0.0205 | 0.206 | 0.0204 |
| | 0.217 | 0.0198 | ... | 0.0211 |
| | 0.202 | 0.0192 | ... | 0.0208 |
| | 0.202 | 0.0194 | ... | 0.0208 |
| | | | ... | 0.0208 |
| 8 | 0.202 | 0.0224 | 0.237 | 0.0284 |
| | | | 0.228 | 0.0286 |
| 9 | 0.215 | 0.0218 | 0.230 | 0.0286 |
| | 0.218 | 0.0211 | 0.233 | 0.0286 |
| | 0.201 | 0.0218 | 0.236 | 0.0276 |
| | | | 0.229 | 0.0279 |
| | | | 0.234 | 0.0280 |
| | | | ... | 0.0281 |

[a] Each pair of figures represents a separately weighed and analyzed portion of ergot. All assays by the same analyst.

were rendered alkaline with sodium bicarbonate, mixed with siliceous earth, and packed in a chromatographic tube. Alkaloid was eluted with chloroform and analyzed by the proposed column separation method. Recoveries ranged from 95 to 98%. In similar experiments, when ergotamine and ergotoxine salts were employed, quantities of alkaloid equivalent to not more than 3% of the ergonovine were found in the water-soluble fraction. These two sets of errors tend to balance each other.

Table II shows the results obtained by several different analysts when using the proposed two-phase shaking extraction and column separation procedure. Agreement between analysts was satisfactory. Several commercial samples of official rye ergot were analyzed by the same analyst using both the N. F. and proposed procedures. The results of these analyses are compiled in Table III. Higher assay results for water-soluble alkaloids invariably were obtained by the proposed procedure. The N. F. requires that the total alkaloid content be not less than 0.15% and that the water-soluble alkaloid content be not less than 0.01%. Samples 2, 4, and 5 do not meet these specifications when analyzed by the N. F. method. All samples meet these minimum specifications when analyzed by the proposed procedure.

The chloroform extracts of several commercial samples were chromatographed on paper. In all cases, the ergonovine spot was more intensely fluorescent than was the ergonovinine spot. The sum of the ergonovine and ergonovinine contents, in some cases, did not seem to equal that of water-soluble alkaloid content by assay. It is assumed that various alkaloid breakdown products are included in the water-soluble alkaloid fraction. Under extraordinary conditions, such as marked changes in temperature from that of ordinary operation, poor resolution may be encountered due to excessive or insufficient movement of the alkaloids. The $R_f$'s should be in the range of 0.2–0.8. If higher $R_f$'s are desired, a less acidic buffer should be employed.

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# Chemical Investigation of *Dioscorea macrostachya* (Benth.)

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**The ultimate analysis of lipoidal matter extracted from the roots and tubers o *Dioscorea macrostachya* (Benth.) disclosed fatty acids present to be palmitic, stearic, oleic, and linoleic. The unsaponifiable matter yielded β-sitosterol, and the presence of unsaturated hydrocarbons, a wax and yellow pigmentary material was indicated. A sterol glycoside isolated was shown to be β-sitosterol glucoside. Proximate analysis for group constituents was also run.**

MAJOR INTEREST in the genus *Dioscorea* during recent years centers about its potential as a source of cortisone-like compounds. Since 1943, several hundred species have been examined for their steroidal sapogenin content by Marker, *et al.* (1), and by Wall and his associates (2, 3). Some of the reports of the latter group include qualitative tests for flavanols, alkaloids, tannins, sterols, organic acids, and phenols. However, there is very little else in the literature on the chemical composition of *Dioscorea* spp. in general and none on *D. macrostachya* in particular. This report deals with a proximate analysis of the constituents of the tubers of this species and with an ultimate analysis of the lipoidal matter isolated from the tubers.

The plant material used in the investigation was obtained from the San Pedro Sula region of Honduras. In 1957, 55 pounds of roots and

Received August 2, 1960, from the University of Connecticut, College of Pharmacy, Storrs.
Accepted for publication August 24, 1960.
Abstracted from a thesis submitted to the Graduate School of the University of Connecticut by William A. Meer in partial fulfillment of the requirements for the Doctor of Philosophy degree.
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