The Spectrophotometric Determination of Ergot Alkaloids. A Modified Procedure Employing Paradimethylaminobenzaldehyde.

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Most of the currently used colorimetric procedures for the determination of ergot alkaloids, including the one official in the United States Pharmacopeia (11), are based on the reaction of the alkaloids with paradimethylaminobenzaldehyde (PDAB). Throughout the history of its development, many modifications of this method has been suggested. Smith (8) who is credited with the first successful quantitative colorimetric procedure, modified the reagent of Van Urk (12) by dissolving PDAB in concentrated sulfuric acid instead of acidified ethanol. With this reagent, he found that exposure to light was necessary for full color development. Allport and Cocking (1) eliminated the necessity for exposure of the reaction mixture to light by adding a small amount of ferric chloride to the reagent. Schlemmer *et al.* (5) stimulated color development by irradiation with ultraviolet light instead of incorporating ferric chloride into their reagent. Silber and Schulze (7) reported, however, that maximum color development could be obtained only when both of these modifications, addition of ferric chloride and irradiation with ultraviolet light, were employed. Schulek and Vastagh (6) on the other hand, employed hydrogen peroxide instead of ferric chloride to enhance color development. Pöhm (4), in a study of the mechanism of the reaction between PDAB and indole compounds, cites a general requirement for dehydrating conditions and for an oxidizing agent when the reaction is conducted in the absence of light and oxygen.

The method employing ferric chloride and official in the USP XVI as well as the method(s) employing ultraviolet irradiation appear to be the most commonly used in present-day research. The former method employs a reagent whose lifetime is limited to 7 days and it requires a one-hour standing period for full color development; the latter methods have a similar limitation on the lifetime of reagents containing ferric chloride, but they require only 7 to 15 minutes for full color development. However, there is an additional requirement for a source of ultraviolet radiation and for vessels capable of holding the reaction mixture in a shallow layer in order to insure adequate irradiation of the entire solution. Experience in this laboratory with the latter-type procedure as described by Stoll *et al.* (9) provided evidence that maximum reproducibility could be achieved only by careful adherence to a set of standard conditions of irradiation. Silber and Schulze (7) gave recognition to some of these factors in their citation of the type of ultraviolet lamp, the distance of the samples from the lamp, the exposure time and other conditions and apparatus used in their assays.

In 1960, Sprince (10) reported that the detection of indoles on paper chromatograms could be enhanced by the application of a spray of 1 per cent aqueous sodium nitrite solution 2–3 minutes after spraying with the PDAB reagent. The sodium nitrite was found to lessen the time of color development and reduce the tendency of the spots to fade. The utilization of this procedure with both paper and thin-layer chromatograms of ergot alkaloids in this laboratory proved to be so superior to other methods that efforts were initiated to adapt this modification to the determination of ergot alkaloids in solution.

EXPERIMENTAL AND RESULTS

Procedure.—The assay procedure representing the method devised in this study was designated the "nitrite" procedure and is conducted as follows:

> To 2.0 ml of a suitably diluted ergot alkaloid solution (containing 5–100 μg total alkaloids) add 2.0 ml of 0.1 per cent PDAB in sulfuric acid–water (1:1 v/v). Mix well and allow to stand for 10 minutes. Add 0.1 ml of freshly prepared 0.1 per cent aqeuous sodium nitrite solution, mix and measure the absorbance at 590 mμ.

For comparative studies, two other procedures, representing methods employing ferric chloride or ultraviolet irradiation, were employed. The USP XVI procedure (11) utilizes a ferric chloride-containing reagent and was performed by adding 4.0 ml PDAB test solution to 2.0 ml sample, allowing the reaction mixture to stand in subdued light for one hour and measuring the absorbance at 590 mμ.[1] The "ultraviolet" method used (9) was conducted by adding 4.0 ml PDAB reagent to 2.0 ml sample, irradiating for 15 minutes with unfiltered light from a Hanovia quartz lamp, model no. 30600 held at a distance of approximately 10 inches from the samples, and measuring the absorbance at 590 mμ.

TABLE 1. *Effect of concentration of sulfuric acid on color development*

Sulfuric acid dilutions[a]		Absorbance	
sulfuric acid ml	water ml	initial	after six hours
3.0	7.0	0.280	0.270
3.5	6.5	0.380	0.350
4.0	6.0	0.400	0.370
4.5	5.5	0.425	0.400
5.0	5.0	0.425	0.390
5.5	4.5	0.420	0.390
6.0	4.0	0.410	0.390
6.5	3.5	0.410	0.370
7.0	3.0	0.360	0.320
7.5	2.5	0.320	0.280

[a]The sulfuric acid, diluted with distilled water in the proportions shown in the table, was used to dissolve the PDAB.

The absorbances of all assay mixtures were measured with a Bausch and Lomb Spectronic 20 spectrophotometer using one-half inch OD colorimeter tubes. Because of the differences in the densities of the samples and the reagents, extra effort was given to insure thorough mixing. Exposure of the alkaloid solutions and the reaction mixtures to strong daylight should be avoided.

PDAB Reagent.—The effect of varying the concentration of sulfuric acid in the PDAB reagent was studied with the following assay system: 2 ml standard ergonovine maleate solution (20 μg/ml), 2 ml 0.1 per cent PDAB in sulfuric acid, and 0.1 ml 0.05 per cent sodium nitrite solution. The sample was mixed with the reagent. Then the sodium nitrite solution was added, the solutions mixed and the absorbance measured by 590 mμ. A second measurement was made after the solutions stood for six hours at room temperature. The results are presented in table 1.

[1]The absorbance was measured at 590 mμ instead of 550 mμ as recommended in the USP procedure in order to permit comparison with the results of the other methods. The results presented in figure 2 show that such a modification is permissible.

Experiments were also conducted to determine the optimum concentration of PDAB in the reagent. Assays were performed with 2 ml PDAB reagent containing 0.05, 0.1 or 0.2 per cent PDAB in sulfuric acid–water (1:1); the absorbances given were 0.40, 0.415, and 0.40, respectively. The procedure described above was used.

Sodium Nitrite Requirement.—The concentration of sodium nitrite required for optimum color development in the assay system was studied by adding 0.1–0.5 ml volumes of a 0.05 per cent sodium nitrite solution. The results are shown in table 2.

TABLE 2. *Effect of concentration of sodium nitrite on color development*

0.05% sodium nitrite solution[a] ml	Absorbance
0.1	0.365
0.2	0.370
0.3	0.370[b]
0.4	0.370[b]
0.5	0.370[b]

[a]Sufficient distilled water to make each addition equal to 0.5 ml was added.

[b]The resultant blue color faded rapidly with the gradual development of a yellow-green color.

TABLE 3. *Effect of time of addition of sodium nitrite*

Time of sodium nitrite addition (minutes after adding PDAB)	Absorbance
0.25	0.160
0.50	0.190
1.0	0.255
2.0	0.350
5.0	0.420
10.0	0.430
15.0	0.440

TABLE 4. *Effect of temperature on required standing time*

Time of sodium nitrite addition (minutes after adding PDAB)	Absorbance	
	3°C	40°C
2.0	0.275	0.420
5.0	0.345	0.420
10.0	0.360	
15.0	0.405	

Time and Temperature.—During the performance of some assays, it was noted that full color development did not occur if the sodium nitrite was added too soon after adding the PDAB reagent. Assays were conducted in which the sodium nitrite solution was added at various time intervals after the addition of the PDAB reagent. In this and in the following experiments, 0.1 ml of 0.1 per cent sodium nitrite solution was used. The results are presented in table 3.

Another variable that remained uncontrolled was that of temperature. It was observed that the heat generated by mixing the PDAB reagent with the sample

raised the temperature of the reaction mixture to about 40°C initially with a subsequent decrease upon standing. In order to examine the influence of temperature, the assay was carried out with the reaction vessel held in a water bath at 3°C and at 40°C. The results are shown in table 4.

Standard Curves.—A number of samples containing various amounts of ergonovine maleate were assayed by the "nitrite" method in order to determine the

TABLE 5. *Calibration data for the colorimetric determination of ergonovine maleate by the "nitrite" method, the USP XVI method and the "ultraviolet" method*

Ergonovine maleate μg	Absorbance		
	"nitrite"	"ultraviolet"	USP[a]
none	0	0	0
19.5	0.180	——	0.147
39.0	0.370	0.207	0.285
58.5	0.577	——	0.420
78.0	0.780	0.438	0.550
97.5	0.958	——	0.690
117.0	1.15	0.668	——
136.5	1.37	——	——
156.0	1.60	0.886	1.09
175.5	——	——	——
195.0	——	1.16	1.34
234.0	——	1.40	1.56
273.0	——	1.68	——

[a]Absorbance measured at 590 mμ instead of 550 mμ as stated in the USP. These values may be converted to those which would be obtained at 550 mμ by multiplying by 1.12.

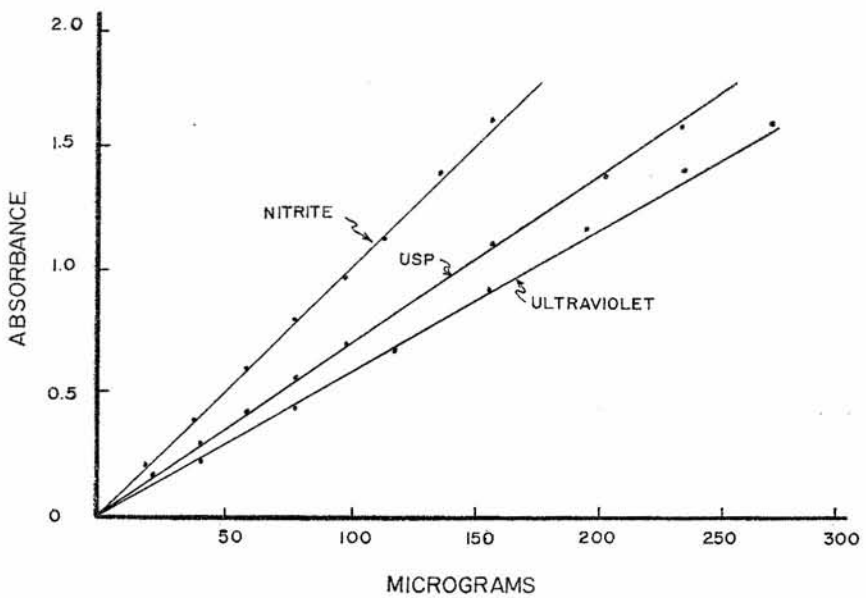

FIG. 1. *Standard curve of ergonovine maleate as determined by the "nitrite" method, the USP method and the "ultraviolet" method.*

range over which Beer's Law is obeyed. Similar determinations were conducted with the USP method (11) and also with the method employing ultraviolet light exposure (9). The results are shown in table 5 and figure 1.

Recovery of Added Alkaloid.—Since the newly developed "nitrite" method would be used primarily for the assay of fermentation broths, it was essential

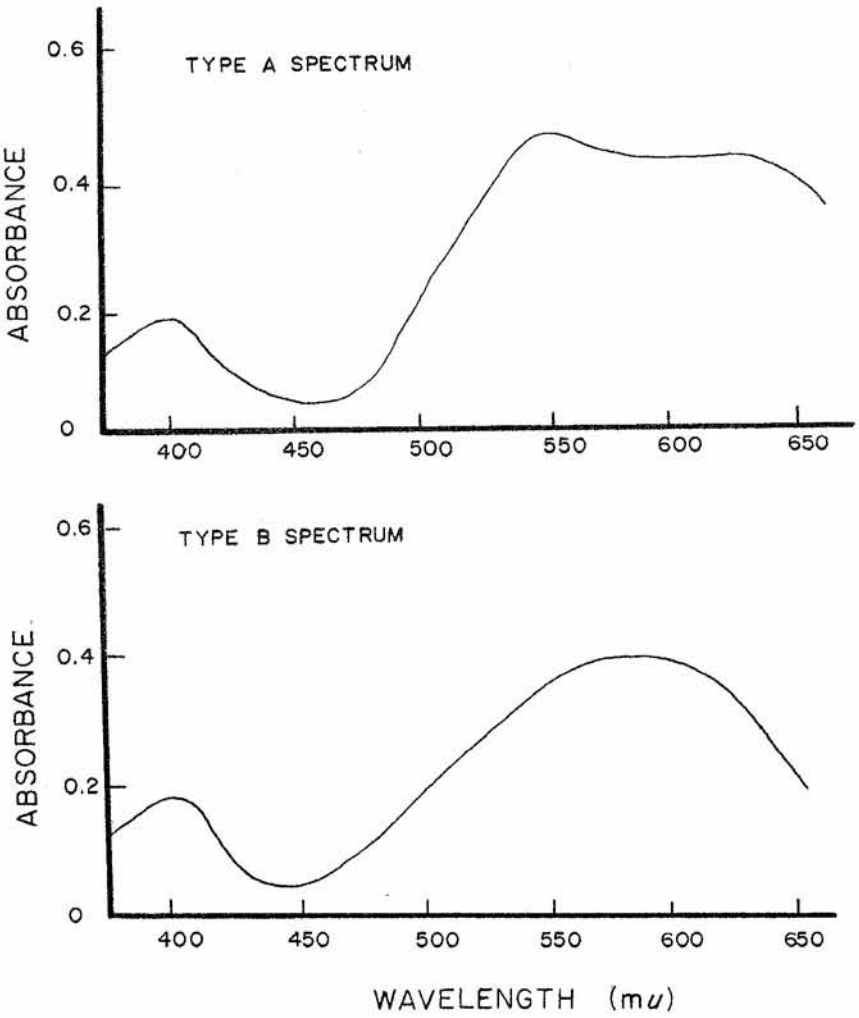

FIG. 2. *Absorption spectra of the reaction products resulting from treatment of ergot alkaloids with PDAB under different conditions.*

that the method be free from interferences by broth constituents. In order to determine whether any interferences existed, a sample from a fermentation was assayed before and after addition of a known amount of ergonovine maleate. Similar tests were conducted with the USP and "ultraviolet" methods of analysis. Results are given in table 6.

Absorption Spectra.—The visible absorption spectra of the colored solutions resulting from the assay of standard solutions of lysergic acid, ergonovine, ergo-

tamine, agroclavine, elymoclavine, and dihydroelymoclavine by each of the three methods of analysis were determined with a Cary recording spectrophotometer, model 14. The results showed that all of these spectra fall into one of two types: these are designated type A and type B in figure 2. The type A spectrum possessed maxima at 405, 545 and 625 mμ; the type B spectrum possessed maxima at 405 and 585 mμ. The type A spectrum was given by lysergic acid, ergonovine and ergotamine when assayed by the "nitrite" method and the USP method. The type B spectrum was given by all of the alkaloids when assayed by the "ultraviolet" method and by agroclavine, elymoclavine and dihydroelymoclavine when assayed by both the "nitrite" method and the USP method.

TABLE 6. *Recovery of added alkaloid from fermentation liquors*

Method	Sample[a]	Additions of ergonovine maleate[b] μg	Alkaloid found as ergonovine maleate μg	Recovery of added alkaloid %
"Nitrite" procedure	1.0 ml filtrate	none	35.7	——
	" " "	19.5	57.5	102
	" " "	none	37.5	——
	" " "	19.5	57.5	102
	1.0 ml filtrate	none	38.5	——
	" " "	19.5	59.0	105
USP XVI procedure	1.0 ml filtrate	none	38.2	——
	" " "	19.5	56.0	91.2
	1.0 ml filtrate	none	39.0	——
	" " "	19.5	58.5	100
	1.0 ml filtrate	none	41.4	——
	" " "	19.5	57.9	84.6
"Ultraviolet" procedure	1.0 ml filtrate	none	54.5	——
	" " "	39.0	97.5	110
	1.0 ml filtrate	none	58.0	——
	" " "	39.0	101	110
	1.0 ml filtrate	none	54.5	——
	" " "	39.0	101.5	120

[a]The samples were culture filtrates obtained from fermentations producing primarily lysergic acid alkaloids (3).

[b]USP reference standard ergonovine maleate was added in a volume of 1.0 ml. Where no alkaloid was added, 1.0 ml of distilled water was added to the sample.

Stability.—The results presented in table 1 show that the intensity of the blue color produced by the "nitrite" assay procedure undergoes very little change after standing for six hours at room temperature.

The PDAB reagent was tested for its capacity to accomplish full color development after storage for one month at room temperature (20–22°C) in both darkness and in laboratory light. The absorbances given by the same standard alkaloid solution were 0.40 with the freshly prepared reagent, 0.415 with the reagent stored in the dark and 0.40 with the reagent stored in the light for one month. At the end of the one month storage, there was no discernible color changes in the reagents themselves. One-month old sodium nitrite was not effective in stimulating production of the blue color. However, solutions up to one-week old retained their capacity to bring about full color development. When a similar experiment was conducted, identical results were obtained with the USP method. The USP reagent showed, however, a considerable darkening of color during the one month storage, especially when exposed to light.

Other Procedures.—Tests were run using 3 per cent hydrogen peroxide to promote color development. The usual procedure of analysis was employed but one drop of hydrogen peroxide solution was substituted for the sodium nitrite solution. When ergonovine standards were assayed, a blue color having the type A spectrum (figure 2) resulted. The intensity of the color, however, was only 75 per cent of that given by the "nitrite" assay at all points on the spectrum.

Hydrochloric acid is the mineral acid used in the chromatographic spray reagent. Considering this, a reagent was made in which the sulfuric acid–water (1:1) was replaced by concentrated hydrochloric acid. This reagent was used to assay an ergonovine standard with the "nitrite" procedure. The results showed that a blue color having the type B spectrum (figure 2) was produced. The absorbance at 590 mμ was approximately 10 per cent higher than that resulting from the standard "nitrite" procedure. On standing for a day, the reagent prepared in hydrochloric acid developed an intense yellow coloration. Because of this apparent instability and the inconvenience of working with a volatile strong acid, hydrochloric acid was eliminated from consideration as a solvent for the PDAB.

Performance of the assay with PDAB dissolved in various concentrations of perchloric acid resulted in failure to develop a blue color. When similar experiments were conducted with phosphoric acid, the intensities of the resulting blue colors were proportional to the concentration of the acid. However, the absorbance obtained with 85 per cent phosphoric acid solution was still only two-thirds of that obtained with the sulfuric acid reagent.

DISCUSSION

The results shown in table 1 clearly indicate that there are limits to the concentration of sulfuric acid that may be employed without a decrease in sensitivity. The greatest sensitivity could be obtained by using a smaller volume of PDAB reagent with a high concentration of sulfuric acid. In this manner, the blue color resulting from the reaction would be diluted to the least extent. Observations of the reagents prepared for this experiment, however, indicated that high concentrations of sulfuric acid caused changes in PDAB that led to a deep yellow coloration. Thus, a limitation on the upper concentration of sulfuric acid permissible in preparing the reagent was imposed by the stability of the PDAB. Considering these results, a 1:1 (by volume) ratio of sulfuric acid–water was selected for preparation of the reagent. Results obtained with the 0.05, 0.1 and 0.2 per cent PDAB reagents showed that the concentration of PDAB was not critical in the range used. The concentration selected for use in the standard assay procedure (0.1%) provides a larger molar excess of PDAB over the highest alkaloid levels that would be assayed by this procedure.

Investigation of the amount of sodium nitrite required for maximum color development revealed that color intensity is limited by very low amounts of the nitrite, while high concentrations produced a blue color which changed to yellow-green. This green color appeared after several minutes when 0.3 ml of 0.05 per cent sodium nitrite solution was added; it appeared more rapidly as the volume of added sodium nitrite solution increased. The dependence of the intensity of the blue color on the amount of sodium nitrite at the low levels examined provides evidence that nitrite is a stoichiometric reactant rather than a catalyst. The use of 0.1 ml of 0.1 per cent solution supplies sufficient sodium nitrite for a blue color that is both fully developed and stable.

The requirement for a standing period after the addition of the PDAB reagent suggests the existence of a noncolor-producing reaction between the PDAB and the alkaloid. This reaction is, however, essential for later color development. Because the rate of this reaction is dependent on temperature, it is not photochemical in nature. The results show that neither the temperature nor the time requirement must be critically controlled within certain limits. With the recom-

mended assay procedure under ordinary laboratory conditions, neither of these factors affect the results of an assay. The observations discussed thus far are consistent with the mechanisms proposed by Pöhm (4) and by Schulek and Vastagh (6).

It can be seen from figure 1, that both the USP and the "nitrite" methods conform to Beer's Law up to an absorbance of approximately 1.5. In contrast, the "ultraviolet" method shows conformance up to an absorbance of 0.9 and becomes very erratic at higher values. Appreciable errors at absorbances greater than 1.5 would be expected because of limitations of the instrument. Similar results were obtained with various other ergot alkaloids.

When each of the assay methods is considered with respect to the entire procedure, the "nitrite" method is the most sensitive. However, when the absorbances are corrected for the dilution resulting from the different final volumes, the "nitrite" and the USP methods give the same values. On the contrary, the "ultraviolet" method remains less sensitive than the other two. The results of Silber and Schulze (7) show that when the "ultraviolet" procedure is conducted with a PDAB reagent that contains ferric chloride, the sensitivity is about 25 per cent greater than that obtained through use of a reagent without ferric chloride. If this correction is applied together with a correction for dilution, the absorbances obtained with the "ultraviolet" procedure used in this study would be very similar to those given by the "nitrite" procedure.

It is frequently necessary in the assay of a solution of an uncommon ergot alkaloid, to express the results on the basis of some readily available alkaloid that was used as a reference standard in the assay. Such is often the case, for example, in the quantitative analysis of alkaloids occurring in fractions issuing from a chromatographic column. The expression of these results could be made more precise in knowledge of the absorbances given by molar solutions of the various alkaloids was available. The situation would be further simplified it if was found that the various ergot alkaloids gave rise to the same molar absorbance; in this case, the expression of the results could be changed simply by multiplying by the appropriate ratio of molecular weights. In table 7, it can be seen that the only similarities in absorbances given by equimolar concentrations of the alkaloids were those shown by the group clavine alkaloids and the pair of lysergic acid alkaloids, ergonovine and ergotamine. Because commercially obtained samples of all of these alkaloids were used, the possibility exists that nonalkaloid impurities in the lysergic acid sample were responsible for its failure to fall into the latter group. The establishment of the purity of these alkaloids was not carried out because it was the intent of this experiment to show only gross differences between the two groups. These results clearly point out that except in the cases where similarity has been established, it is not permissible to convert results obtained with one reference standard to some other standard by correcting only for molecular weights.

The excellent recoveries obtained with the "nitrite" method showed that there are no serious interferences when this method is used in the analysis of fermentation broths. The high recoveries obtained with the "ultraviolet" method and the low recoveries obtained with the USP method indicate some type of interference in these cases.

Examination of the type of spectrum given by the various alkaloids shows that the lysergic acid alkaloids give rise to the Type A spectrum (figure 2) and the clavine alkaloids give rise to the Type B spectrum (figure 2) when assayed by the "nitrite" and the USP methods. Both types of alkaloids give rise to the type B spectrum with the "ultraviolet" method. Considering this, 585 mμ would be the most useful wavelength in the assay of a mixture containing both clavine and lysergic acid alkaloids. Approximately 10 per cent higher absorbance can be obtained when determining lysergic acid alkaloids at 545 mμ with the methods employing sodium nitrite or ferric chloride. However, the use of this wavelength

would be a distinct disadvantage in the assay of mixed alkaloid samples. Also, at this lower wavelength more interference is given by the yellow-brown color of fermentation broths and many natural extracts. The visible absorption spectra of the color given by the reagent of Allport and Cocking (1) with agroclavine, triseclavine, penniclavine and lysergene was measured by Yamatodani (13) and the differences were used to determine the structural type to which the alkaloid belonged. The latter three alkaloids give a yellowish green color with this reagent. The blue color given by various ergot alkaloids upon treatment with *p*-toluenesulfonic acid (2) appear to fall into the two types described in this study. Although the entire spectra are not shown, the values presented for the position of the peaks indicate a close similarity to those found in the present work.

TABLE 7. *Molar absorbances of the blue color given by various ergot alkaloids*[a]

Alkaloid[b]	Absorbance/Micromole
Ergonovine maleate	4.41
Ergotamine tartrate	4.39
Lysergic acid	3.92
Elymoclavine	5.63
Dihydroelymoclavine tartrate	6.01
Agroclavine	5.70

[a]The values presented represent the absorbance (measured at 590 mμ) that would be given by one micromole of the alkaloid in the "nitrite" assay mixture. Each value represents the average of three determinations.

[b]The alkaloids were crystalline commercial samples used without further purification. In every case, chromatography with various systems showed only one alkaloid component.

The PDAB reagent used in the "nitrite" method has excellent stability and gives satisfactory results even after one month storage in the light or dark. The sodium nitrite solution, however, can be effectively used only up to periods of one week if stored in a stoppered container. In order to avoid failure of the assay, it is recommended that this solution be freshly prepared.

The blue color produced by the "nitrite" assay is stable in ordinary laboratory conditions for at least six hours. Exposure of the colored assay mixture to direct sunlight hastens the fading of this color. Exposure of the alkaloid solution to direct sunlight prior to the assay causes changes; in this case, a decreased absorbance. Such exposure, therefore, should be avoided during the course of the assay.

CONCLUSION

The newly developed "nitrite" method possesses the following advantages over other methods: greater sensitivity, great stability of the PDAB reagent and more rapid performance time. Its only disadvantage is the requirement that the sodium nitrite solution be freshly prepared.

SUMMARY

A spectrophotometric procedure based on a modification of the method employing paradimethylaminobenzaldehyde (PDAB) for the determination of ergot alkaloids has been developed. The procedure utilizes sodium nitrite to enhance the color produced by the reaction between PDAB and the alkaloid. Studies comparing this procedure with the USP XVI method and with a procedure employing ultraviolet light to promote color development shows that the new method pos-

sesses advantages over existing methods with respect to sensitivity, speed of performance and stability of reagent.

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