Table VIII.—Antipyrine-Phenacetin

No compound; eutectic at 62% antipyrine with m. p. of 76° C.

Mol % Antipyrine	Thaw Point, ° C.	Melting Point, ° C.
0.00	136	137
8.12	78	131.5
20.87	76.5	122.5
25.91	76	120
39.73	76	105
46.86	76	97
59.03	76.5	81
72.55	76	91
78.30	76	97
90.05	76	106.5
100.00	111	112

Table IX.—Mandelic Acid-Betanaphthol

No compound; eutectic at 53% mandelic acid with m. p. of 88.5° C.

Mol % Mandelic Acid	Thaw Point, ° C.	Melting Point, ° C.
0.00	121.5	122.5
9.90	91	117
19.65	90	111.5
30.35	88.5	105.5
39.94	89	99.5
50.49	89	91
59.91	88	96.5
71.26	88	105
79.64	88	109.5
90.78	90	115
100.00	117	119

Table X.—Mandelic Acid-Phenacetin

No compound; eutectic at 56.5% mandelic acid with m. p. 79° C.

Mol % Mandelic Acid	Thaw Point, ° C.	Melting Point ° C.
0.00	136	137
9.96	79	130
19.92	79	123
30.00	79	115
39.06	79	106
49.35	79	92
60.01	79	84.5
70.86	79	97.5
80.30	78.5	106
87.05	78.5	112
100.0	117	119

Table XI.—Mandelic Acid-Sulfonal

No compound; eutectic at 57% mandelic acid with m. p. of 86° C.

Mol % Mandelic Acid	Thaw Point, ° C.	Melting Point, ° C.
0.00	125	126
9.97	88.5	122
19.72	86	118.5
30.11	86	112
39.74	86	105
50.14	86	96
59.78	86	90.5
69.89	86	98.5
78.36	86.5	105.5
90.53	87.5	113.5
100.00	117	119

Table XII.—Betanaphthol-Phenacetin

No compound; eutectic at 61% betanaphthol with m. p. of 70° C.

Mol % Betanaphthol	Thaw Point, ° C.	Melting Point, ° C.
0.00	136	137
10.09	71	131
19.78	70	124
29.79	70	114
40.26	70	103
50.05	70	91
60.48	70	72
70.15	70	89
79.61	70	103
89.50	72	114
100.00	121.5	122.5

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(1) Rheinboldt, H., *J. prakt. Chem.*, 111 (1925), 242; Rheinboldt, H., and Kircheisen, M., *Ibid.*, 112 (1926), 187; *Ibid.*, 113 (1926), 199, 348.

Report on the Vanadium Oxytrichloride Colorimetric Method for the Determination of Capsaicin in Capsicum

By Alice Hayden and C. B. Jordan**

In July 1933 L. F. Tice (1) published in the *American Journal of Pharmacy* an article on "A Simplified and More Efficient Method for the Extraction of Capsaicin Together with the Colorimetric Method for Its Quantitative Determination in Capsicum Fruit and Oleoresin." The colorimetric assay as described in the above publication was later modified as follows (2):

ASSAY PROPER

"The sample to be assayed would be representative of the whole lot of capsicum and not having an abnormal ratio of any one of the fruit parts. It should be powdered and dried in a desiccator to remove moisture.

"Shake 1 Gm. of the dried and powdered capsicum with 50 cc. of dry acetone in a dry glass-stoppered flask, allow the mixture to stand with occasional agitation for from one-half to one hour and filter.

"Add from 0.2 to 0.3 per cent of dried, non-pungent paprika to about 100 cc. of dried acetone, in another flask, shake it well and filter. Then adjust the color to match that of the acetone extract of the capsicum already prepared, diluting it with sufficient acetone. Use this paprika extract to prepare the standard, by dissolving 7 mg. of capsaicin, accurately weighed, in 50 cc. of the colored acetone.

* Purdue University School of Pharmacy.

This standard consists of a 0.014 per cent solution of capsaicin.

"Pour 5 cc. of the clear capsicum-acetone extract (2 per cent w/v) into each of four matched test-tubes and 5 cc. of the standard solution into another matched test-tube. To the tube of standard solution add 14 drops of a fresh, 1 per cent solution of vanadium chloride ($VOCl_3$) in dry carbon tetrachloride and then 12 drops of the same reagent to the first of the unknowns, 14 drops to the second, 16 drops to the third and 18 drops to the fourth tube. Use a bulb medicine pipette in dropping the reagent.

"Allow the tubes to stand for two minutes, when at least one of the four tubes should develop a greenish blue color equal to or of greater intensity than that observed in the standard tube.

"The standard solution of capsaicin slowly decomposes and therefore must be prepared freshly as needed. All moisture must be absent in every step of the process."

The above method seemed to offer the possibility of a more scientific and accurate evaluation of capsicum than the U. S. P. X organoleptic test. The Chairman of the U. S. P. Sub-committee No. 6 therefore asked several investigators to consider the method to determine whether or not it could be used as a satisfactory test to be recommended for adoption by the U. S. P. Revision Committee. The following persons were asked to collaborate in this study:

Dr. George E. E'we, Tailby-Nason Company
Dr. Joseph Rosin, Merck & Company
Dr. W. L. Scoville, Parke, Davis & Company
Mr. R. E. Himelick, Purdue University

Dr. E'we reported his findings as follows:

"The two lots of capsicum which we have on hand each required 0.3 cc. to 140 cc. of sweetened water to allow the detection of pungency when swallowed. Since the U. S. P. X test specifies that 0.1 cc. will produce a sensation of pungency these capsicums are below the U. S. P. standard. These capsicums were from widely different sources and three individuals confirmed the degree of pungency.

"The Vanadium Chloride method of assay confirmed, in general, the organoleptic tests. The clear-capsicum-acetone extract had to be brought to a 4% strength solution (the initial colors of the extract and the standard capsaicin solution being the same) in order to obtain approximately the same intensity of bluish color in both extract and standard. Both samples of capsicum gave about the same results.

"The number of drops of Vanadium Chloride solution used influences the intensity of the color, the color increasing as the Vanadium Chloride solution is increased. The shades of bluish color yielded by the extracts and by the standard were totally different, the extracts giving greenish blue while the standard gave a 'truer' blue color.

"It would appear that the Vanadium Chloride assay method is a useful qualitative test for capsaicin and for the rough comparison of capsicum , but in view of the widely different quality of colors yielded by the extracts as against the standard and because of the variation in intensity induced by varying the amount of reagent added, its adoption by the U. S. P. would likely be productive of no more uniformity among analysts than the present test."

Concerning the assay, Dr. Rosin writes as follows:

"The colorimetric assay, while it is much shorter, has many pitfalls. In order to make the colors matchable paprika has to be used, and because of the change of the color to green with a slight excess of the vanadium oxytrichloride, several controls have to be made, etc., all of which complications do not recommend themselves very strongly. However, I may be wrong, and I hope I am. At any rate I am prepared to try out the colorimetric assay as you suggest."

Unfortunately, Dr. Rosin was not able to secure sufficient materials to perform the laboratory tests at that time.

Three samples of Mombassa capsicum, together with a sample of paprika, were supplied to Dr. Scoville for his investigations. These same samples were also used for the investigations carried on at Purdue University.

In a communication, Professor Scoville reports:

"Mr. R. T. Harris of the analytical laboratory at Parke, Davis & Company has carried out the Tice method for the determination of capsaicin in capsicums on the three samples of capsicum sent. I witnessed the tests, and since we are in agreement as to the applicability of the test, this is a joint report thereon.

"The three samples of capsicums were first tested by the present organoleptic method, and each responded to the test at a 1 to 70,000 dilution. Five men took the test in each case and all were in agreement. The paprika responded to the test at a 1 to 2000 dilution.

"The three samples to be tested varied considerably in color, numbers 1 and 2 being light in color and gave a light yellow color in acetone solution. Number 3 was darker, more reddish, and the paprika had a reddish brown cast.

"The standard acetone solution of paprika matched fairly closely that of number 3 but had a more brown shade. For the other two, the paprika was diluted one-half in order to get comparable results. But the brownish shade of paprika compared with the yellow of the other two made color comparisons difficult. On adding the vanadium oxytrichloride to them, the paprika showed a distinctly different color from that of numbers 1 and 2—the latter having a greenish tint which was lacking in the paprika. This made color comparisons difficult and much less satisfactory. Each test was made by comparing the paprika solution containing 14 drops of vanadium test, with the 12, 14, 16 and 18 drops in the capsicum solutions.

"In each case the 14-drop tubes showed the closest agreement in color after a minute or so of standing. After about 5 minutes, the 18-drop tests had faded to a green and except for depth seemed to agree more closely in shade with the 14-drop paprika test.

"The color fades rather rapidly, but this could be managed if the initial shades could be more closely adjusted so as to give more satisfactory comparisons. It seems a little unfortunate that the three samples of capsicums were all of the same strength in regard to capsaicin content, and so corresponded in the test. At least, we could detect no difference, either by the organoleptic or the Tice test.

"As a trial of the method it would have been more satisfactory if the capsicums had varied markedly in strength and so given a better indication of the different colors to be expected from different strengths. As we observed them, the differences between the colors as observed from 14 drops and 16 drops of the reagent, while clearly distinguishable, were not sufficiently marked to promise satisfactory determinations of capsaicin.

"We did not have a medicine dropper, so used a 1-cc. graduated pipette for measuring. It was difficult to regulate the finger pressure to count exactly the number of drops in so low tension a liquid as acetone, and we were not sure of our count in all cases—as when two drops or even three fell in rapid succession when only one was intended, so in the second test we dropped in the stated number of drops and also measured the liquid. The results were disconcerting—as follows:

14 drops = 0.17 cc.
16 drops = 0.16 cc.
18 drops = 0.19 cc.
12 drops = 0.15 cc.
14 drops = 0.15 cc.

Evidently we did not get the variations in the amount of vanadium solution that were intended, and this hits a vital spot in the assay.

"On the third sample we did not count the drops but measured 0.15 cc. into the paprika-capsaicin standard and 0.13, 0.15, 0.17 and 0.19 cc., respectively, into the unknowns. The results were practically uniform with the other two tests.

"As stated before, we found each of the three capsicums to correspond most closely to the 14-drop or 0.15 cc. of vanadium reagent. This would, according to Tice's calculations, make each of them a U. S. P. standard capsicum—corresponding to a 1: 70,000 capsicum.

"In its present state the test is more complicated and tedious than the official organoleptic test and we do not feel that the results

are as satisfactory. We have more doubt about the end-point and would feel less certain of variations. Probably this is due in part to the fact that we were comparing a test which is new and one with which we are thoroughly familiar and have had years of experience.

"The principal objections to adopting the Tice method for the Pharmacopœia, as we see it, is, first, the difficulty of matching the shades of the solution which is being determined with that of the standard. Capsicums, even of the same species, vary widely in color. That a color comparison is more accurate than taste-matching I do not question, provided that we have the same tints to match. But when the tints differ the determination of color intensity of one color in a mixture is difficult."

Mr. Himelick made the following observations on the assay:

"1. All three samples of the Mombassa capsicums apparently met the U. S. P. standard (0.7%). Sample No. 1 was estimated to contain 0.9% capsaicin, and samples No. 2 and No. 3 to contain 0.8% capsaicin by the vanadium method.

"2. Sample No. 3 in acetone solution had a darker, reddish color than Samples No. 1 and No. 2. It was more readily matched by acetone colored with paprika than Samples No. 1 and No. 2.

"3. The colors produced with the reagent begin to fade within a very few minutes.

"4. One drop of $VOCl_3$ solution per 0.001% of capsaicin in the standard tube does not produce the darkest blue color when an ordinary medicine dropper is used. This is highly misleading, since a substandard sample may appear to be satisfactory by this method, simply because its deepest color has been produced, whereas in the standard tube it has not.

"5. Using a more dilute $VOCl_3$ solution (say, 0.5%) and measuring it from a small burette, should improve results.

"6. I find that by preparing just one tube of unknown and one standard tube the difference in color produced is distinguishable when the tubes differ by 0.002% or more in capsaicin content (corresponding to 0.14% in crude drug), using skylight for illumination. Color experts claim northern skylight to be superior."

In view of the above findings, the colorimetric assay for capsaicin in capsicums was not recommended for adoption by the U. S. P. Revision Committee. However, it was thought that, with further study, the test might be developed and so modified that it would offer a simple and accurate method by which it would be possible to determine whether capsicums met the U. S. P. requirement (0.7 per cent capsaicin).

In order to eliminate certain difficulties and possible sources of error that had been pointed out by previous workers, and to establish the reliability of the test, a study of the colorimetric assay was undertaken with the following objectives in mind:

1. To definitely determine the time of maceration required to completely extract the capsaicin from a given sample of capsicum.
2. To determine the effect of the paprika solution on the assay.
3. To determine the effect of concentration and quantity of the reagent on the color produced.
4. To provide a permanent, non-fading color standard which would eliminate: (*a*) the difficulty and necessity of preparing capsaicin; (*b*) errors due to the instability of the capsaicin solution.
5. To determine the effects of adulterants on the assay method.

DETERMINATION OF CORRECT MACERATION TIME TO INSURE COMPLETE EXTRACTION OF CAPSAICIN IN CAPSICUM

Six domestic samples of capsicum were used in the following tests: A series of samples was weighed out (1 Gm./50 cc. of dry acetone) and maceration was allowed to take place for the following periods: eight hours, four hours, two hours, one hour and 30 minutes. On a given sample, for example, Sample No. 1 (Louisiana Sun Longs, 1935 crop), the macerations were designated (8), (4), (2), (1) and (30). All comparisons on the same sample were made at the end of the eight-hour period.

Using 5 cc. filtered portions of the acetone extracts with equal quantities of the vana-

dium reagent (quantity previously determined by the number of drops of reagent required to produce the deepest blue color), no perceptible difference could be noted in the color produced with the reagent whether the samples were macerated for 30 minutes or eight hours. Apparently the time as specified in the modified Tice assay (30–60 minutes) is sufficient for extraction of the capsaicin with acetone.

Using ether and alcohol in various extractions, however it was noted that the color depth of the extracts was increased as the length of maceration increased. This was thought to be only an indication of further pigment extraction. Using acetone, the color of the extracts varied only slightly with the longer maceration periods.

PAPRIKA

The paprika used in the preparation of the standard capsaicin solution was tested for pungency by means of the organoleptic test. Out of seven individuals taking the test, only two reported perceptible sensation on the throat with a 1.2000 dilution.

A 1-gram. sample of the paprika was macerated for one hour in 50 cc. of dried acetone. Five cc. portions of this filtered acetone extract were tested with the reagent. Only a few drops of reagent were required to produce a green coloration. In every instance the color was distinctly green—no blue color being apparent even on the addition of the first one or two drops of reagent. The green color faded very quickly —in all instances within five minutes.

Using the 0.2% to 0.3% solution of paprika in acetone for preparing the standard capsaicin, it was a fairly simple matter to match the yellowish amber colored capsicum extracts. It was noted, however, that upon the addition of the reagent, it was sometimes very difficult to make satisfactory color matchings between the standard tube and the unknowns. This difficulty had been noted by previous workers and was expressed by Dr. E'we as follows: "The shades of bluish color yielded by the extracts and by the standard were totally different, the extract giving greenish blue while the standard gave a truer blue color."

As has been pointed out before, the green color obtained by the reagent with the paprika solution fades very rapidly, and the color obtained with capsaicin alone is blue. In order to obtain the best results, the reagent should be added to the unknowns first (since the color seems to persist longer with the capsicums) and the comparisons should be made as soon as the reagent has been added to the standard tube of paprika-capsaicin solution.

EFFECT OF CONCENTRATION AND QUANTITY OF REAGENT UPON COLOR PRODUCED

Vanadium oxytrichloride is a sensitive reagent and in a 1 per cent solution it is frequently difficult to determine the exact end-point of the color reaction (greatest depth of blue color without an excessive greenish cast). Since a single drop of the reagent is capable of producing quite a marked color change, it is suggested that the reagent should be made up quantitatively to insure uniform concentration. A more dilute solution of the reagent (0.5%) enables one to distinguish the end-point more readily.

The use of a small burette or a graduated pipette for adding the reagent in place of the medicine dropper and measuring the quantity of the reagent gave results which were much more readily duplicated than the dropper method. However, in using this type of apparatus one must take precaution to avoid unnecessary exposure of the reagent to air or moisture since it shows the unstable character of the pentavalent vanadium halides in that it undergoes rapid hydrolysis and thereby becomes coated with red flakes of vanadium pentoxide.

In order that different workers may secure more concordant results, it seems pertinent that the reagent should be quantitatively prepared in more dilute solution (0.5%). The inaccuracies introduced by the use of non-uniform medicine droppers and by counting the number of drops added may be eliminated by the use of a small burette or a graduated pipette for adding the reagent.

Attempts were also made to determine if more permanent and more readily comparable results could be obtained using the paper or spot test methods, but these offered no improvement over the tube method.

ATTEMPT TO PROVIDE A NON-FADING COLOR STANDARD

Authorities usually consider that a satisfactory color test should provide colors which are stable and matchable for a period of at least twenty minutes. As has been previously stated, the reagent ($VOCl_3$) produces a blue color with capsaicin. A greenish blue color may be obtained with the use of a paprika-capsaicin solution but the green color fades very rapidly, thus making the comparison with the unknowns extremely difficult unless the comparisons are made immediately following the addition of the reagent to the standard tube. An attempt was therefore made to produce a permanent, non-fading color standard in order to:

(1) increase the period of time in which reliable color matchings of the standard with the unknowns could be made;
(2) eliminate the difficulty and necessity of preparing capsaicin;
(3) eliminate possible source of error due to instability of the capsaicin solution.

The following colorimetric solutions (see U. S. P. XI, p. 557) were prepared: Cobaltous Chloride, Ferric Chloride and Cupric Sulfate. Some of the colors produced under Green (*M*, *N*, *O*) were satisfactory as matches of the standard capsaicin tube. The colorimetric solution of copper sulfate did not afford a blue color of sufficient intensity to be used, so a solution of twice the strength was prepared. This, together with a very small quantity of the ferric chloride solution, produced a fairly satisfactory match of the capsaicin-paprika standard tube. The addition of cobaltous chloride was not necessary and did not seem to affect the color sufficiently to warrant its addition.

EFFECT OF ADULTERANTS ON THE ASSAY

Using paprika, it was pointed out that a 1-Gm. sample of the material used for making the colored acetone (non-pungent paprika), when macerated with 50 cc. of dried acetone, gave a quickly fading green color when a few drops of the reagent ($VOCl_3$) were added to the clear filtrate. In Tice's (1) original article he says, "The use of a dried acetone w/v extract of paprika as the solvent in preparing the standard was found to result in a closer matching of the unknowns with the standards, the reason being that the $VOCl_3$ reacts with the coloring principles of both capsicum and paprika similarly."

A check of the literature disclosed that previous workers had noted other color reactions with the reagent, gingerol and vanillin being mentioned specifically. A number of crude drugs were selected, some because they were known to possess pungency and others simply because they were closely related to drugs which gave color reactions with the reagent, and 1-Gm. samples were macerated for 30 to 60 minutes with 50 cc. of dried acetone. Color reactions were noted with the following when the reagent was added to the clear filtrates:

Ginger
Cloves
Tumeric
Uva Ursi
Sassafras

No color change was noted with the clear filtrates of the following upon the addition of the reagent:

Curry Powder
Black Mustard
Anise
Fennel
Cubeb Berries
Cardamon Seed
Zeodary

The colors produced varied considerably with the drug and the quantity of the reagent added. Using some drugs, however, it was possible to approach very closely the color produced with an acetone extract of capsicum or with the paprika-capsaicin-acetone solution and the reagent. For example, ground drugs which gave no test at all with the reagent (see list above) could be treated with a very small quantity of ground ginger so that the acetone macerations produced a filtrate, which, with the reagent,

gave color tests which approximated the standard capsaicin color very closely. Using paprika or a sub-standard capsicum, it was a very simple matter to "doctor" the sample so that it gave a color which would indicate a standard capsaicin content.

Noting that the crude drugs giving a color reaction with the reagent all contained phenols or phenolic compounds as the principal constituents, other phenols were dissolved in acetone and tested with the reagent. Color reactions were produced with the following:

Phenol
Guaiacol
Hydroquinone
Resorcinol
Vanillin
Thymol
Salicylic Acid
Oil of Bay
Oil of Pimenta
Oil of Cardamon
Oil of Thyme
Oil of Cloves
Oil of Nutmeg
Oil of Chenopodium

Here again, the colors varied considerably, depending upon the concentration of the phenol and the amount of reagent added. Some of the colors produced faded in a short time—the green colors seeming to fade more rapidly than the blue. The color of the standard tube could be so closely matched by some of these phenols that it was impossible to distinguish the tube containing capsaicin from the one which was capsaicin-free.

Of course, it is realized that not all of these substances could be satisfactorily used as adulterants. Most of the oils, for example, could be quite readily detected by their odors. In other instances, a careful microscopic examination in conjunction with the organoleptic test would probably detect an adulterated sample. However, the mere fact that an acetone extract of a given sample, when treated with the reagent, produced a color which would match a standard capsaicin-paprika-acetone solution with the reagent, would not suffice as a satisfactory qualitative test for capsaicin in capsicum and hence could not be developed into a satisfactory quantitative method of evaluating capsaicin content.

The reaction of vanadium oxytrichloride with phenols in acetone solution seems to be quite a sensitive test and might be developed as a satisfactory generic method of distinguishing this group of compounds.

CONCLUSIONS

1. The maceration period of 30 to 60 minutes is sufficient to extract the capsaicin from capsicum using acetone as the solvent.
2. The duration of the reliable color in the standard is not long enough to insure satisfactory matchings with the unknowns.
3. A permanent, non-fading color for the standard may be achieved by using colorimetric solutions of cupric sulfate and ferric chloride.
4. More concordant results are obtained by using a weaker solution of the vanadium oxytrichloride (0.5 per cent prepared volumetrically), and measuring the reagent from a graduated pipette or a burette instead of using the dropper method.
5. A number of substances containing phenolic compounds gave color reactions with the reagent. In some instances, the colors produced approximated the standard so closely that a color test using vanadium oxytrichloride would not serve as a reliable qualitative or quantitative test for capsaicin.
6. Further investigation might prove vanadium oxytrichloride to be a sensitive generic test for phenolic compounds.

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(1) Tice, L. F., *American Journal of Pharmacy*, 105 (1933), 320.
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"Never utter these words: 'I do not know this, therefore, it is false'. One must study to know; know to understand; understand to judge"—APOTHEGM OF NARADA