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A MODIFIED VOGES-PROSKAUER METHOD
OF DETERMINING DIACETYL
IN DAIRY CULTURES

BY

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University

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OF DETERMINING DIACETYL
IN DAIRY CULTURES

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable as meeting the thesis requirements for this degree, but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Thesis Adviser Date

Head, Dairy Science Department Date

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INTRODUCTION

Dairy scientists have known for a number of years that diacetyl is one of the principle flavor components of butter, buttermilk, and cheese cultures. Of the tests which have been introduced to determine diacetyl, one of the first was the heavy metal gravimetric method. This procedure involved distillation of the diacetyl from the mother substance. The most common derivative formed was nickel dimethylglyoximate. Principle disadvantages of this method were that large amounts of sample were required for steam distillation when the products were low in diacetyl. It was also a time consuming procedure because the steam distillation could take as long as 1 hr followed by a 24 hr period for the dimethylglyoxime derivative to precipitate.

It has been known for a number of years that the speed and accuracy of colorimetric determinations were far superior to the gravimetric method. All attempts to perfect a reliable colorimetric test had failed until Prill and Hammer (21) introduced their method in 1938. Other procedures have been introduced since that time, however none has been used as extensively. Some of the tests introduced were direct methods of estimation. These methods usually deleted the steam distillation and color was developed from the original sample. It was necessary to filter the sample to obtain a color complex relatively free from impurities. It has been common knowledge that a direct analysis would shorten the time that was inherent in the steam distillation methods.

Anantharamaiah *et al.* (1) used the modified Voges-Proskauer reaction to estimate diacetyl quantitatively. They attained some degree of success with this direct method. It was the purpose of the research reported herein to ascertain if the modified Voges-Proskauer method introduced by them was capable of yielding reproducible results, or if certain modifications are necessary.

LITERATURE REVIEW

The quantitative tests for the detection of diacetyl in cultured dairy products are numerous. One of the first used was the gravimetric method, as described by Michaelian and Hammer (14). This was a steam distillation method to determine diacetyl and usually acetylmethylcarbinol, as a nickel dimethylglyoximate derivative. Some problems were encountered, such as low results when small amounts of diacetyl were being assayed and a prolonged time to run the test. The reasons stated above prompted a number of research workers to develop colorimetric methods.

Prill and Hammer (21) introduced a colorimetric reaction that had a high degree of accuracy as well as being capable of determining diacetyl in amounts as low as 0.001 mg in 5 ml of water. This method used steam distillation, similar to the gravimetric procedure. The diacetyl that was driven from the original sample by steam was collected in a test tube containing 1 ml of equimolar quantities of hydroxylamine hydrochloride and sodium acetate. This reagent converted the diacetyl to dimethylglyoxime when heated in an 85 C water bath for 1 hr. Excess hydroxylamine oxidized the ferrous iron in alkaline conditions; the former being assimilated with the addition of acetone dipotassium phosphate. The red color of the reaction is based upon the formation of an ammonoferrous dimethylglyoximate after the addition of the developing reagents. These reagents consisted of concentrated ammonium hydroxide, saturated potassium sodium tartrate and ferrous sulfate. After filling the test tubes to 10 ml with distilled water and inverting to enhance color stabilization, the tubes are read either in colorimetric or photometric equipment.

Pien <u>et al</u>. (19) used a steam distilling apparatus for determining diacetyl in dairy products. By reacting the diacetyl with diaminobenzidine, a derivative termed diphenylguinoaline was formed, which gave a yellow color. These investigators claimed that amounts as low as 0.5 mg of diacetyl per kg of butter could be detected when working with 100 g samples. Using the Pien <u>et al</u>. method, Cox and Wiley (4) refined it so that the amount to be analyzed could be reduced in size. By using a less fragile fractionating column, smaller samples and smaller quantities of reagents, the accuracy of the test was improved. The authors claimed comparable results with the modified test and with the gravimetric nickel dimethylglyoxime method. The technique was very useful where speed and simplicity were important in analyzing a large number of samples.

Englis <u>et al</u>. (6) described a method for the determination of diacetyl in dairy products. They converted diacetyl to dimethylglyoxime and measured the ultraviolet absorption at 226 mμ in a spectrophotometer. Acetylmethylcarbinol does not interfere with the absorption at this wavelength. Direct determination of diacetyl in butter without preliminary distillation was not successful.

Owades and Jakovac (17) introduced a method for diacetyl detection which differed from the Prill and Hammer method only in the procedure used to distill the diacetyl into hydroxylamine. The tube containing the culture was immersed in a 65 C water bath. Nitrogen gas was used to transport the diacetyl into the hydroxylamine hydrochloride to form dimethylglyoxime. Pack *et al.* (18) modified the Owades and Jakovac method so 12 samples could be assayed simultaneously. These authors claimed greater accuracy, sensitivity and simplicity for this gas distillation method. Less expensive equipment was used and it was possible to run a large number of samples at one time.

Ruehe and Corbett (23) developed a volumetric method for the determination of diacetyl. They added ferric chloride to the culture to convert the acetylmethylcarbinol to diacetyl and it was then steam distilled. Sodium hydroxide was used to neutralize the distillate to the phenolphthalein end point. Hydrogen peroxide and osmic acid were added to oxidize the diacetyl. After standing for 3 hr the solution was titrated to the faint pink end point of phenolphthalein with sodium hydroxide. The method is based on the reaction that hydrogen peroxide will oxidize one mole of diacetyl into two moles of acetic acid.

Diacetyl determination in other products. Hill and Wenzel (10) developed a colorimetric test for the detection of diacetyl and acetylmethylcarbinol in orange juice. The method was based on the Voges-Proskauer reaction. They used steam distillation to separate the diacetyl from the juice. The color development was accomplished by adding 5 ml α-naphthol and 2 ml of KOH containing creatine. The tubes were allowed to stand for 10 min for color development. The authors stated that the reaction time was of the utmost importance, because the color decreased steadily after reaching maximum intensity.

Beisel *et al.* (3) used the Hill and Wenzel modification of the Voges-Proskauer reaction on California Valencia orange juice. They reported the detectable limits of diacetyl to be 0.05 ppm, acetylmethylcarbinol to be 0.5 ppm and 2,3,-butylene glycol to be 40,000 ppm.

Fields (8) used the Voges-Proskauer test to determine acetylmethylcarbinol in apple juice. This was used as a basis to detect low quality raw material. High levels of acetylmethylcarbinol are indicative of bacteriological growth on the apples prior to processing.

Komm and Flugel (12) developed a quantitative test for the detection of diacetyl in bread. The method had a sensitivity of about 0.0001 ppm. The unknown solution was compared to a standard diacetyl solution. They used *m*-dinitrobenzoic acid in absolute alcohol to produce a wine red color in an alkaline solution.

Voges-Proskauer color reaction. The Voges-Proskauer test is based on the production of a red color complex at the surface of certain bacterial cultures. The red color is produced by the addition of a strong alkali to the culture. Barritt (2) discovered that by the addition of α-naphthol to the Voges-Proskauer test he obtained a greatly intensified color, with increased sensitivity and without any loss of specificity. He stated that he had no evidence concerning the nature of the reaction; but thought it could be forming a compound with a diacetyl-guanido combination. He also showed that α-naphthol must be added to the reaction prior to the addition of alkali. If it was added later no enhanced color change took place.

Prill et al. (20) referred to the Voges-Proskauer reaction and stated that diacetyl reacts within a short time to give a wine red color. Acetylmethylcarbinol is oxidized to diacetyl under the conditions of the test. They also reported that methylglyoxal, n-amylglyoxal, di-n-butyryl and alicylic diketones gave no color, but in some cases a brown color was produced by the alkali alone. A slight color was produced from dipropionyl, while methylphenyl diketone produced a very deep color. They also stated that many of the metal derivative tests had limitations because they did not show exact quantitative diacetyl determination in the presence of α-dicarbonyl compounds. This also applied to derivatives of various osazones or quinoxalines.

Eggleton et al. (5) stated that Voges and Proskauer observed a pink color in broth cultures of certain bacterial species after alkali had been added. The test had very little application as a quantitative test until Barritt used α-naphthol to enhance the color reaction. For the first time it was possible to use this reaction for the quantitative estimation of creatine or diacetyl. Their work confirmed Barritt's findings and they observed that the reaction took place at room temperature. It was also observed that color started to develop at the surface, suggesting that oxygen was involved in the reaction. The reaction was studied in the absence of oxygen and no color developed for a period of 4 hr. As atmospheric oxygen was admitted, the color began to develop immediately, and it attained the same color intensity as the control.

A Warburg monometric apparatus was used to measure the ml of oxygen taken up during the reaction. Two ml of alkali or 2 ml of 1% α-naphthol solution in stock alkali were placed in the main chamber of the manometer cup. The creatine and diacetyl solutions were placed in the side arm.

> "By this means the following points were established (for a temp. of 26): (1) Creatine in alkaline solution does not absorb O_2. (2) α-naphthol in alkaline solution consumes O_2 at a slow but steady rate over a period of many hours. (3) Diacetyl in alkaline solution shows a rapid initial O_2 uptake which falls off, sometimes completely after 30 min. It amounts to about 1 mol. O_2/mol. diacetyl. (4) On mixing diacetyl with α-naphthol in alkali there is a consumption of O_2, the rate of which is approximately equal to the sum of the rates of O_2 consumption of diacetyl and α-naphthol separately. (5) The addition of diacetyl and creatine to the alkali causes a rapid consumption of O_2 for the first 30 min., which falls off to a low steady rate. The presence of excess of creatine increases by 2-or-3 fold the amount of O_2 consumed by diacetyl during this 30 min. In other words, O_2 is involved in the Voges-Proskauer reaction, though it is not possible to say how much, since under the conditions of these experiments some of the diacetyl must have oxidized without reacting with creatine. (6) When diacetyl and creatine are added to α-naphthol in alkaline solution, there is an initial rapid phase of O_2 consumption lasting about 30 min., followed by a slow steady O_2 uptake lasting over a period of hours. The amount of O_2 used by this mixture is greater than when creatine is omitted, indicating that the modified Voges-Proskauer reaction also involves an oxidation by molecular O_2. (7) The rate of O_2 consumption is approximately equal to the rate of colour development. The rate of O_2 consumption was obtained as the difference between the rate of O_2 consumption of a mixture of diacetyl, creatine and α-naphthol and the rate of O_2 consumption of a similar mixture of diacetyl and α-naphthol."

The color development was measured and was within the limits of experimental error. Owing to the speed of the reaction and side reactions the error was large, but color development and oxygen consumption ran parallel. Their experiments showed that both the Voges-Proskauer and Barritt reactions involved an oxidation by molecular oxygen. This fact was given attention when using the method for the determination of either creatine or diacetyl.

Eggleton _et_ _al_.(5) used the term pigment for the compound responsible for the red color. Attempts to isolate the "pigment" failed because they could not obtain the compound in a crystalline state. The product has been known to remain stable for 4 yr when in the solid state. In solutions a red color was present at pH 10, purple at pH 8, and brown at pH 6 with slight solubility at pH 4. These facts suggested that the compound had an isoelectric point at pH 4 with three ionizing groups. In concentrated acids, as hydrochloric or sulfuric, the compound was purple in color and quite stable. The optimum pH for maximum color intensity was investigated. The Barritt reaction showed no color development when sodium bicarbonate was used to make the solution alkaline. Red color development was enhanced when either sodium carbonate or sodium hydroxide were used. The deepest color was obtained when a mixture of these two chemicals were used. The alcoholic α-naphthol solution recommended by Barritt tended to become dark in color after standing. Maximum color development of the α-naphthol was rather intense. Diacetyl in excess caused a yellow color which masked the red color; whereas a deficiency of diacetyl caused substandard color production. The color was proportional to the amount of creatine present. However, when creatine was in excess the color was proportional to the amount of diacetyl present. This showed that there is a linear relationship between the intensity of color and the amount of diacetyl. The chromogenic powers of creatine and diacetyl are approximately equal. This may indicate that the red pigmented compound could be in equimolar proportions with diacetyl and creatine.

Many guanido compounds react according to Barritt's method. Eggleton _et_ _al_. (5) examined arginine, guanidine and glycocyamide, but these produced about one-ninth of the color produced by an equivalent amount of creatine. Creatine phosphate and creatinine will not give a color test if freshly prepared. They reported that by using trichloracetic acid as a protein precipitant it can interfere with the reaction of the alkaline α-naphthol solutions. A blue pigment which masks the red color of the normal reaction is produced in the presence of ultraviolet light. Trichloracetic acid does not give the blue color when the Voges-Proskauer reaction is carried out in a dark room or in the absence of ultraviolet light. Ammonia was also reported to be responsible for reduction of the red color. Glycine, β-alanine and β-alanyl histidine all showed an inhibiting tendency. The substances mentioned above inhibited the color production by diacetyl in the presence of excess creatine. This indicated that the interference of the reaction was not a destruction, but rather a reaction with some intermediate compound in the Voges-Proskauer reaction.

Westerfeld (24) suggested a simpler and more sensitive test than the gravimetric nickel dimethyglyoxime method. He perfected a colorimetric method for the determination of blood acetoin. It was based on the original color reaction produced by the Voges-Proskauer reaction plus the addition of α-naphthol. He stated that the original Voges-Proskauer reaction was not sensitive or reproducible; but the Barritt modification made it a satisfactory quantitative test. The colors obtained were reproducible but somewhat unstable. Maximum color was obtained for diacetyl, between 5 and 10 min after the addition of α-naphthol and KOH. He reported the color obtained with acetoin was more stable and that maximum intensity was reached in 1 hr after the addition of α-naphthol and KOH. Loss of color was observed to be as high as 10% in diacetyl in 30 min.

Guanido compounds such as arginine, creatinine, guanidine carbonate and methylguanidine sulfate gave a color complex in the presence of diacetyl, α-naphthol and KOH. The maximum color was obtained with creatine. He also stated that α-naphthol could not be replaced by any of a large number of substances he tested and it was of little value to add the α-naphthol after the KOH.

Westerfeld (24) prepared a standard diacetyl solution containing 1 mg of diacetyl per 1 ml of water. The diacetyl was purified by fractional distillation at 88 C. Acetoin was prepared from the crystalline polymer and was purified by washing with anhydrous ether or by fractional distillation. The standards were kept for several months at 6 C; however, diacetyl deteriorated before acetoin. The color was developed in a series containing from 1 to 12 ppm of acetoin or diacetyl and read against reagent blanks. Standard curves for both diacetyl and acetoin were linear for amounts below 5 to 6 ppm. However, the lines deviated from the standard curves when larger amounts were assayed. He reported that the Voges-Proskauer test was nearly specific for diacetyl or acetoin; however, 5 carbon ketols gave the same color as diacetyl while the 6 carbon ketols gave a light olive green color within 10 min. Nickel dimethylglyoxime was more specific for diacetyl than the colorimetric reactions in most tests. He also stated that the possibility existed that unrelated substances may react with α-naphthol.

Ennor and Stocken (7) showed that sulphydryl or disulphide compounds can interfere with the Voges-Proskauer reaction. This could be overcome by the addition of p-chloromercuribenzoic acid to the reaction. With the addition of p-chloromercuribenzoic acid it was possible to assay for phospho-creatine. Most substances that contain -NH groups were generally considered to be inhibitors. Tests were run on a few biological and nonbiological compounds. Of these piperidine, glycylglycine, iminodiacetic acid, diketopiperazine, sarosine, proline and hydroxyproline acted as inhibitors. It was considered conceivable that an accumulative effect of small amounts of various peptides and amino acids, as well as sulphydryl and disulphide compounds, could act as inhibitors. They had trouble with different inhibitors with different samples. This difficulty was overcome by using the Eggleton _et_ _al_. method of increasing sensitivity by increasing the concentration of the α-naphthol.

Some additional work to substantiate Eggleton's _et_ _al_. method showed that of the compounds tested, only di-methylguanidine reacted in the same manner as creatine. Whereas the reactions of guanidine, arginine, glycocyamine and methylguanidine showed no indication of reaching completion in a determinable color level in 50 min. To obtain the same color intensity at 30 min the latter compounds mentioned needed a ten fold increase in concentration.

Rosenberg _et_ _al_. (22) reported a modification to the Voges-Proskauer reaction which enabled them to estimate the amount of arginine in protein hydrolysates. The color was developed in the presence of n-propanol and a 1% solution of α-naphthol. The sensitivity of the test was increased to the point where the color developed from monosubstituted guanidines was about equal to that obtained with disubstituted guanidines. A number of alcohols were investigated for the color intensity of the Voges-Proskauer reaction. It was found the n-propanol gave the greatest intensification of color whereas ethanol was rated second. They also reported that maximum color development was obtained at 20 min with n-propanol and ethanol, but if the alcohol was omitted the time for maximum development was extended. Heating the reaction in a water bath tended to stabilize the color. The optimum time-temperature relationship appeared to be at 30 C for 15 min for this reaction. A straight line was obtained when the time required to reach maximum color development was plotted against temperature.

These workers also showed the color development end point of various guanidines. Creatine tended to have the most activity either in the absence of n-propanol or at a low concentration of α-naphthol. Arginine had a high extinction coefficient when n-propanol was used in the reaction, but creatine tended to have a higher value. They also showed that other compounds present in protein hydrolysates did not exert any large degree of inhibiting effect on color reaction. They were able to recover 100% ±4% of the arginine present in the hydrolysate. Diacetyl and α-naphthol in alkaline solutions reacted with guanidines possessing a free guanido group ($-NH_2$). Guanidines of the general formula similar to creatine produced a color five to ten times as intense as the monosubstituted ones. Many of the simple alcohols increased the sensitivity of the reaction. The nature of the chemical reaction of the alcohols is not known; however, it is known that the alcohols increased the speed of the development of the red color. They also tended to have a stabilizing effect on the color.

Macpherson (13) introduced a modified colorimetric method that he felt was advantageous in many respects to other tests. He stated that in the estimation of small quantities of material, the colorimetric method was superior to gravimetric analysis. Also, the colorimetric procedure was simpler and quicker to perform, which is an important factor when a large number of samples are to be analyzed. He stated that the method used must be reliable, specific, sensitive and capable of giving consistent results. However, these conditions are not always fulfilled with the colorimetric method. After experimenting with both α-naphthol and acetylbenzoyl, he decided that the colorimetric method had a better chance for modification. The procedure which he thought gave the best results had the addition of urea before the hypobromite, thus the color was built up in two stages. Consistent and maximum colors were developed, which were stable for a considerable time.

<u>Voges-Proskauer reaction applied to lactic cultures</u>. Zarins and Brants (25) perfected the first method for the determination of diacetyl in dairy products which eliminated the distillation process. A 30 ml sample of a lactic acid culture was filtered to obtain the clear whey. Sodium hydroxide was added to 10.0 ml of the whey, and after 15 min a precipitate formed which was removed by filtration. Two and one-half ml of the filtrate were treated with 0.5 ml of a 1% creatine solution and 2.5 ml of a 25% solution of KOH. The mixture had to react for 2.5 hr with stirring at one-half hr intervals. Direct sunlight was found to affect the reaction. The test could be shortened to approximately 1 hr by adding small portions of a 1% $CuCl_2$ solution. Another method of shortening the reaction was by reducing the amounts of whey serum, creatine and KOH to 1.0, 0.1, 1.0 ml respectively. The authors claimed close agreement for their method when compared to the gravimetric procedure.

Hammer (9) investigated the possibility of using the Voges-Proskauer test as a rapid examination of lactic acid cultures for acetylmethylcarbinol and diacetyl. He used equal volumes of a lactic culture and 40% NaOH with a small amount of creatine to obtain the color reaction. The amount of the red color complex at the surface was directly proportional to the amount of acetylmethylcarbinol and diacetyl present in the culture. He concluded that this was a rapid screening test that could be employed to determine the diacetyl and acetylmethylcarbinol content in lactic acid cultures.

King (11) modified the Voges-Proskauer test so it could be used for determining acetylmethylcarbinol and diacetyl in lactic acid cultures. He used 30% KOH in the Hammer creatine test for detection of acetylmethylcarbinol and diacetyl. The α-naphthol reagent that he used consisted of 0.1 g dicyandiamide, 4.0 g α-naphthol, 10.0 ml amyl alcohol, and 50.0 ml ethyl alcohol. He recommended that an analytically pure α-naphthol be used, which tended to retard a dark discoloration of the reagent. Dicyandiamide appeared to give a clear color reaction in all tests. If a culture containing the whole protein fraction was used, dicyandiamide may be omitted; however, in serum, filtrate or distillate, dicyandiamide or creatine was essential for the color reaction. The color intensity was dependent upon the amount of α-naphthol used. Increased quantities of α-naphthol gave an enhanced red color. He also reported that oxygen from the air entered into the reaction. After 30 min of reaction time a red-lilac color was obtained. This color was compared with the color intensity of a known standard.

Anantharamaiah *et al.* (1) applied the Voges-Proskauer reaction to the direct estimation of diacetyl in cultured milk. The content of diacetyl in bacterial cultures estimated by the new colorimetric method agreed closely with the gravimetric procedure. Color production was proportional to the concentration of diacetyl in the range of 1 to 10 ppm. A linear relationship was maintained in the presence of impurities from the skimmilk. The order in which the reagents were added is very important. Sensitivity of the test is decreased by as much as 90% if the α-naphthol is added after the alkali.

EXPERIMENTAL PROCEDURE

The modified Voges-Proskauer method used in this investigation was derived from a number of workers. One of the first tests introduced was the Voges-Proskauer method. In the Voges-Proskauer test, alkali was added to the cultures which produced a pink color on the surface after a length of time had elapsed. The pink color was produced when arginine or creatine reacted with diacetyl in the presence of a strong base. Acetylmethylcarbinol had to be oxidized to diacetyl before any color reaction took place. O'Meara (15) introduced the use of creatine to enhance the development of this pink color. Barritt (2) added α-naphthol to the reaction, which increased the sensitivity of the test and made it possible for the test to be used quantitatively.

Preparation of cultures. Lactic acid cultures from the culture collection of the Dairy Science Department at South Dakota State University were used in the tests. The cultures were carried in whole milk which was steam treated at 98 C for 45 min and cooled to 21 C prior to transfer. They were transferred on a Monday, Wednesday and Friday schedule. The length of incubation time depended upon the activity of the culture, from a minimum time of 13 hr to a maximum time of 18 hr. The cultures were then removed from the 21 C incubator and placed in a 5 C refrigerator until used.

Preparation of blank. The steamed whole milk used for the blank was acidified with dilute HCl to a pH of 4.4. It was treated in the same manner as the lactic acid cultures except that it had no bacteriological growth. The above procedure was used on all blanks that were used for diacetyl determination, either by the modified Voges-Proskauer or the Prill and Hammer methods.

Anantharamaiah determination of diacetyl. The reagents were prepared according to the Anantharamaiah *et al*. (1) method. A 15% solution of trichloracetic acid was used to deproteinize milk cultures. The material to be checked was adjusted to a pH of 6.0. The solutions were filtered through Whatman No. 40 filter paper. A 1.0 ml portion was pipetted into a test tube and 0.1 mg of arginine, 0.6 ml of a 5% solution of α-naphthol and 0.2 ml of 30% KOH were added in that order. After 8 min of color development the contents of the tubes were diluted to 10 ml and well shaken. The tubes were placed in a boiling water bath for 2 min, cooled to room temperature and read for color intensity in a photoelectric colorimeter. By using this method the optimum wavelength was obtained. These workers found minimum interference of light transmission at a wavelength of 490 mμ. Standards were determined by using 1 ml portions of sterile skimmilk containing 0.5 to 10 g per ml in the final dilution.

Modified Voges-Proskauer determination of diacetyl. The reagents for this method were prepared as follows:

1. Creatine . . . . 1 mg/ml of double distilled water
2. α-Naphthol . . . 5 g/100 ml of 95% ethyl alcohol
3. KOH . . . . . . 30 g/100 ml of double distilled water

Analytical grade reagents were used on weight/volume basis.

A 10 g sample of lactic acid culture was weighed into a 50 ml beaker on a triple beam (Ohaus) balance. The reagents were added in this order: 1 ml of double distilled water, 1 ml of creatine reagent, 6 ml of alcoholic α-naphthol and 2 ml of KOH. It is very important that they be added in this order to insure maximum color development. A 6 min reaction period was allowed before they were filtered. Folded filter paper (Balston No. 12) was used for filtering the sample. After the KOH had reacted for 20 min, a 1 ml portion of the filtrate was transferred into a cuvette and diluted with double distilled water to a total volume of 10 ml. The contents of the cuvette were mixed by inversion. The cuvette was placed into a 95 C water bath for 5 min, then it was removed from the hot water bath and cooled to room temperature.

Prill and Hammer determination of diacetyl. The reagents were prepared according to the Prill and Hammer (21) method. With the exception of steam distillation this method was used to standardize a stock solution of diacetyl for the preparation of standard curves. The original method was used as a standard of comparison for the modified

Voges-Proskauer method in determining the amounts of diacetyl in lactic acid cultures. Diacetyl from the stock solution and the steam distilled diacetyl from the cultures were added to tubes containing 1 ml of equimolar quantities of hydroxylamine hydrochloride and sodium acetate. The diacetyl was converted to dimethylglyoxime after heating in an 85 C water bath for 1 hr. While the tubes were still warm, 1 ml of acetone dipotassium phosphate was added to counteract the effect of excess hydroxylamine. Ammonium hydroxide was added to each tube in 0.3 ml portions. Saturated potassium sodium tartrate reagent was added in 2.2 ml amounts per test tube. The last reagent added was 0.2 ml of ferrous sulfate. The volume was increased to 10 ml with double distilled water. The tubes were inverted to enhance the intensity and stability of the color. Maximum color was not obtained for at least 15 min; however, the color was read within 2 hr before the color faded.

Color measurement. The intensity of color produced by the modified Voges-Proskauer and the Prill and Hammer methods were read after the cuvette was cooled to room temperature. The optical density was read in a Bausch and Lomb Spectronic 20 photoelectric colorimeter at the optimum wavelength.

Preparation of standard diacetyl solutions. A standard solution of diacetyl was made by pipetting 1 ml of diacetyl (Fisher Reagent Chemical Catalog No. D-18) into 1 liter of double distilled water.

A series of dilutions were made containing calculated amounts of 8.8, 4.4 and 2.2 ppm of diacetyl. One ml of the standard diacetyl solution (88 ppm) was pipetted into a 10 ml volumetric flask. The flask was filled with 9 ml of 20 C double distilled water. The flask then contained the 8.8 ppm dilution. A 4.4 ppm dilution was made by taking 5 ml from the 8.8 ppm dilution, pipetting it into a 10 ml volumetric flask and filling it to 10 ml. The 4.4 ppm dilution was diluted further by pipetting 5 ml into another 10 ml volumetric flask and filling to 10 ml. The last dilution contained 2.2 ppm of diacetyl. The diacetyl content in the series of dilutions was determined by the Prill and Hammer method.

Determination of standard curves. Standard curves were made with the standardized stock solution containing 88 ppm diacetyl. By using different increments of diacetyl and developing color by the Modified Voges-Proskauer Method, a standard curve was prepared by the following procedure. Ten g portions of HCl acidified (pH 4.4) whole milk were weighed into 50 ml beakers. Stock solutions of diacetyl were added in 0.0, 0.2, 0.4, 0.6, 0.8 and 1.0 ml portions to as many beakers of acidified milk. Water was added in 1.0, 0.8, 0.6, 0.4, 0.2, and 0.0 ml increments to the beakers. This provided an equal dilution factor to each ml of acidified milk. Color was developed by the Modified Voges-Proskauer Method as stated above. The optical density of the red color complex was plotted on graph paper. The slope value of the curve was determined by reading the point of interception at 8.8 ppm diacetyl against the optical density line at this point. The value of this point multiplied by the optical density gave the concentration of the unknown diacetyl of the lactic acid cultures.

RESULTS AND DISCUSSION

Numerous tests have been introduced for the detection of diacetyl; however, only a few methods have been developed which eliminated distillation procedures. Some of the early attempts to detect diacetyl by direct analysis ended in low recovery or in complete failure. Many workers have used the Voges-Proskauer reaction as a qualitative test for the determination of diacetyl in milk products.

Anantharamaiah _et_ _al_. (1) were the first to use the method quantitatively for lactic acid cultures. The various procedures that they used to achieve their color reaction and optical density readings were followed. These workers used a wavelength of 490 mu because they found minimum interference from the impurities in the tubes.

Optimum light transmittance. The optimum light transmittance was checked with a standard solution containing 6 ppm diacetyl. This solution was chosen because it was near the middle of the useable color range. There was a low color intensity at 2 ppm diacetyl and a high intensity at 10 ppm diacetyl. The tube with 6 ppm diacetyl was checked for optimum optical density after the galvanometer was nulled with the blank. Minimum and maximum wavelength capacities of a Bausch and Lomb Spectronic 20 photoelectric colorimeter were explored for optimum optical density readings.

Figure 1 shows that the optimum wavelength was at 540 mμ. The difference in wavelengths between this work and that of Anantharamaiah et al. (1) could be attributed to the degree of impurities in the filtrate.

Use of trichloracetic acid. Anantharamaiah et al.(1) used 15% trichloracetic acid to deproteinize their milk cultures. When this procedure was followed in this laboratory a blue color became quite intense. This blue color and a flocculent precipitate made the optical density readings very erratic. The flocculent precipitate appeared in the trichloracetic acid filtrate after the addition of 9 ml of water to bring the total volume to 10 ml in the cuvette. The precipitate could be dispersed by shaking the tubes; however, it settled to the bottom of the tube in a short time. The addition of an excess of KOH did not alter the structure of the floc. Adjusting the pH from 10 down to 8 changed the color from red to purple and to brown at pH 6. The blue color that was mentioned above tended to mask the red pigment in the tubes. The solutions at the lower concentration of diacetyl were more severely masked than the solutions with the higher concentrations of diacetyl.

Eggleton et al. (5) reported the same color changes of the compound when they lowered the pH. They also studied the effect of trichloracetic acid as an interfering agent in the Voges-Proskauer reaction. They reported that the masking blue color was caused when the reaction was carried out in the presence of ultraviolet light.

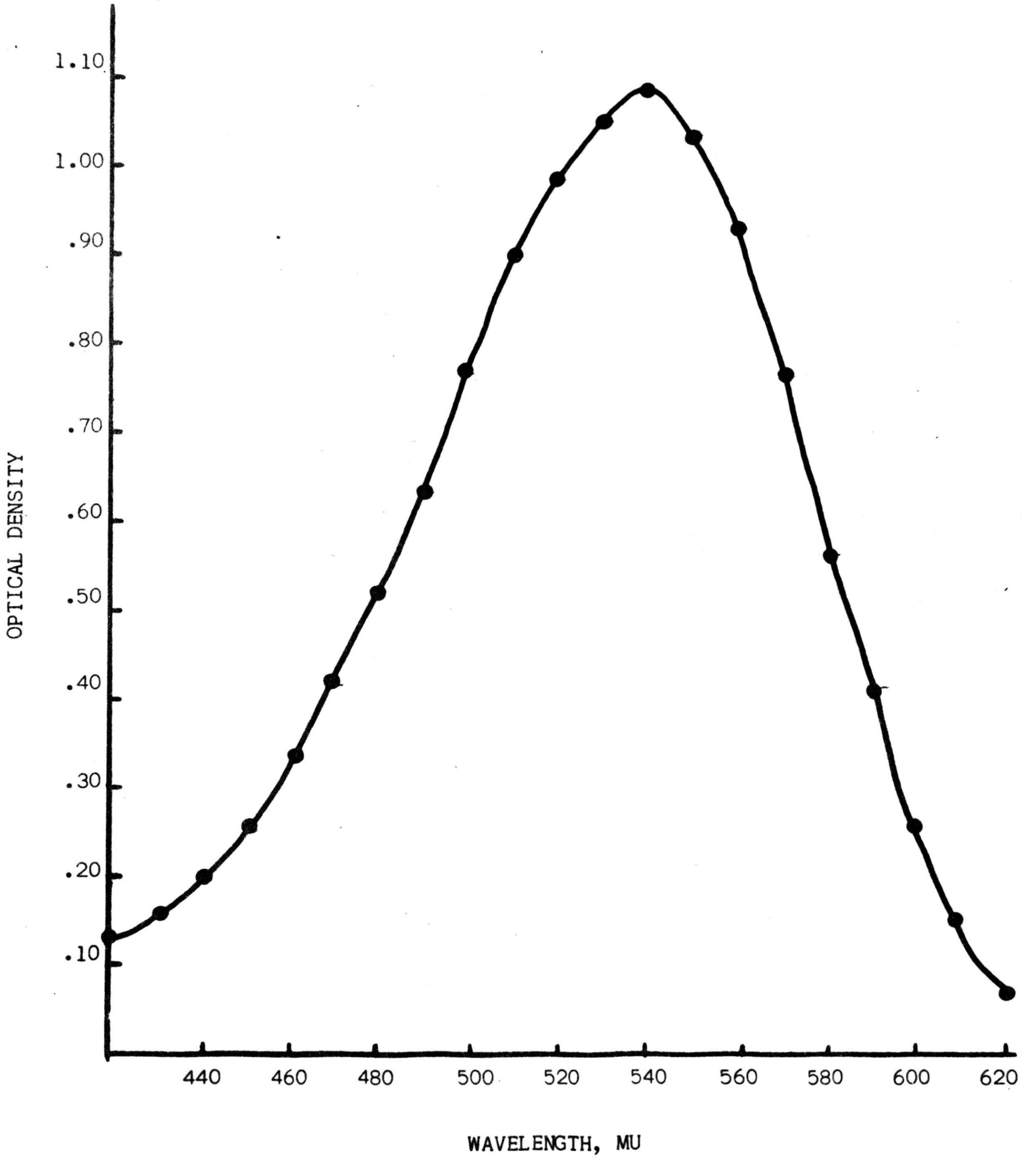


Figure 1. Optimum wavelength for red color complex.

However, the color defect could be controlled if the reaction was carried out in a darkroom or in the absence of ultraviolet light.

Figure 2 shows some of the erratic readings obtained when trichloracetic acid was used. The tests were run in duplicate and read at wavelengths of 540 mμ and 490 mμ. Anantharamaiah _et al_. (1) reported the least interference at a wavelength of 490 mμ. The optical density readings from the duplicate samples that were read at the 540 mμ wavelength tended to have a greater variance than the same samples when read at the 490 mμ wavelength. Duplicate samples that were read at the higher wavelength showed a maximum deviation of 4 points on the optical density scale. At the lower wavelength, the maximum deviation between duplicate samples was 3 points. The angle of the slope was less in the graph with the lower wavelength which tended to reduce the difference between points in the optical density readings. The erratic readings that were obtained when trichloracetic acid was used to deproteinize the culture, proved that some other approach would have to be used to obtain a clear color reaction. A clear filtrate was obtained when KOH was added to the culture after α-naphthol, and no flocculent precipitate appeared when water was used as the diluting agent.

Diluting agents. Rosenberg et al. (22) used different diluting agents to increase the color intensity of the modified Voges-Proskauer reaction. Some of the diluting agents were tested. Ethyl alcohol was used to dilute 1 ml of the red pigment to 10 ml quantities; however,

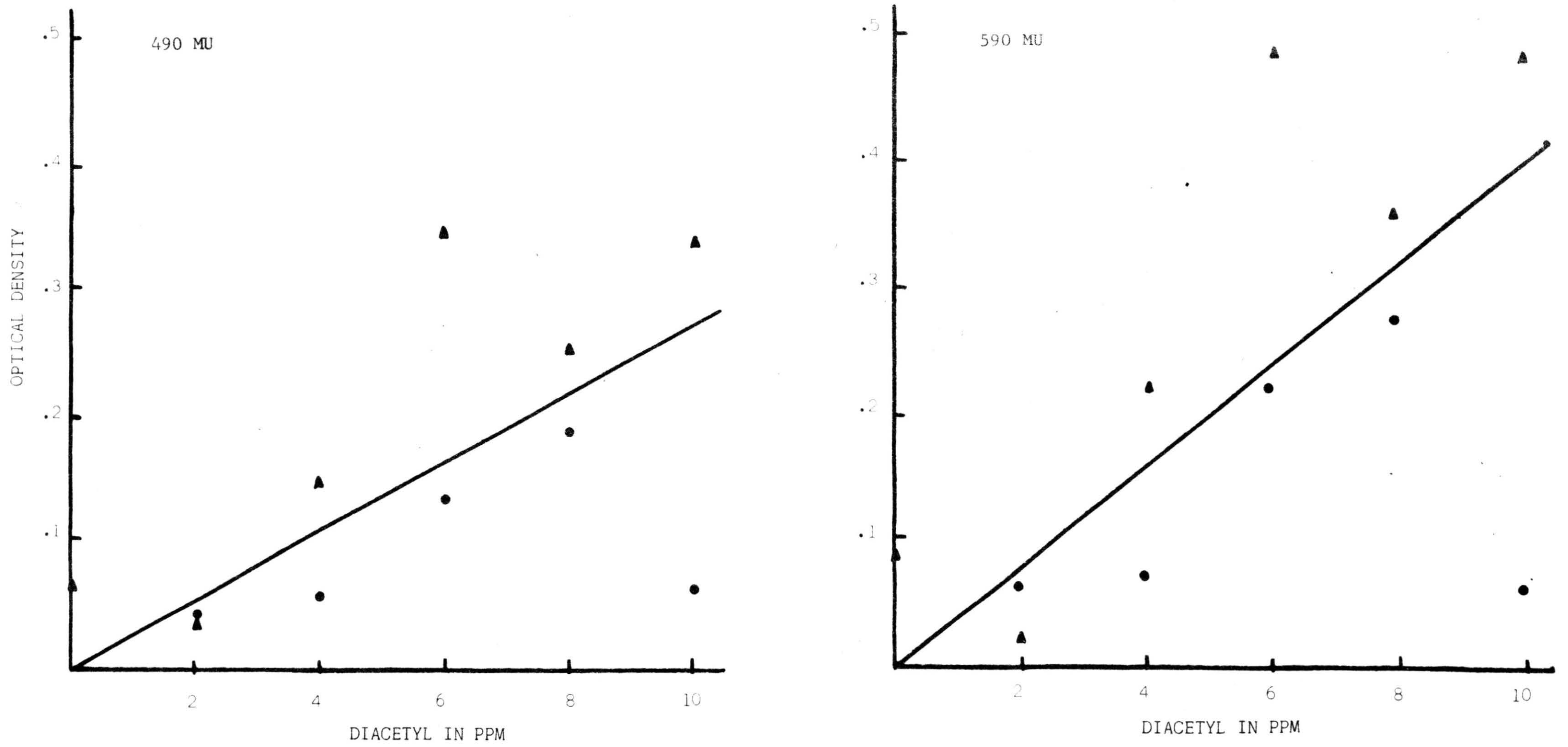


Figure 2. Comparison between 540 mμ and 490 mμ wavelengths.

▲ Trial 1. ● Trial 2.

the ethyl alcohol precipitated the soluble proteins in the alkaline (pH 10) filtrate. The red color may have been enhanced but with the flocculent precipitate it was impossible to read the optical density. A cloudy milky colored precipitate was obtained when n-propyl alcohol was used. The degree of difference between the tubes was impossible to read because of the heavy milky color brought about by the addition of n-propyl alcohol. Methyl alcohol was used for a diluting agent also, but it had the same undesirable attributes that the other alcohols had.

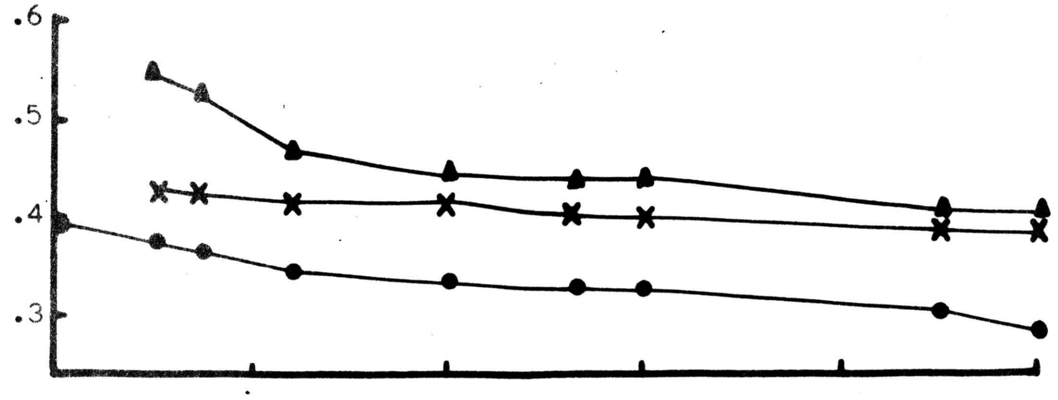
Double distilled water was used in the cuvettes containing the red pigment; two tubes were used, arginine in one and creatine in the other. There was no precipitate of any nature when double distilled water was used; however, the tubes with creatine gave a brighter red color than the tubes containing arginine. With the water as the diluting agent the tests could be duplicated; whereas, when the other solvents were used the degree of error was greatly increased.

Comparison of creatine with arginine. Creatine was used in the tests because it gave a brighter red color than arginine. The enhanced color could be attributed to the shorter chain structure of creatine as compared to arginine. Arginine is part of the casein molecule and can be cleaved off the protein chain by acid hydrolysis and may enter into the reaction. The amount of free arginine that may react in the color reaction could vary from one culture to another. The brighter red color that creatine produced, when it was part of the reaction, tended to complement the optical density readings of the test.

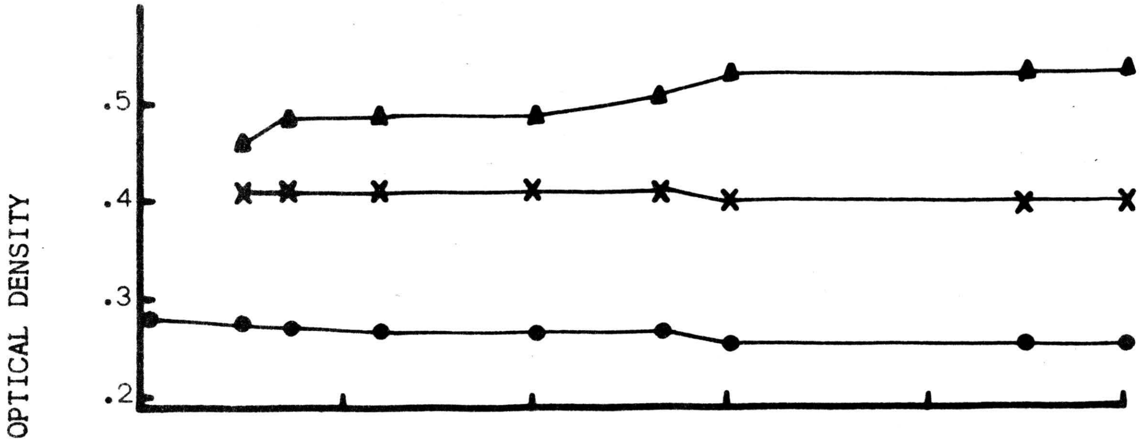
Use of α-naphthol. The chemical condition of the α-naphthol is very important in the Voges-Proskauer reaction. The α-naphthol in stock was a number of years old. It had oxidized until it was dark brown in color. When a 5% (w/v) solution of α-naphthol and 95% ethyl alcohol was prepared, it was dark brown in color. When this was used for the Voges-Proskauer test, very erratic results were obtained. A new supply was obtained, (J. T. Baker Chemical) which was light brown in color in contrast to the older α-naphthol. The new supply of α-naphthol was a very light brown in color when it was made up to a 5% (w/v) solution. It also produced a more consistently clear red filtrate than the old reagent had done. After the alcoholic solution of α-naphthol was made, it was placed in the freezing compartment of a refrigerator. Storage of this compound in a cold dark place retarded the oxidation of α-naphthol as well as retarded the alcohol from taking up water. This in turn prolonged the life of the reagent, and enabled a larger quantity of alcoholic α-naphthol to be made up at one time. The stock solution was stored, as stated above, for 6 wk before the color started to darken. This time probably could have been prolonged, but the stock solution was exposed to the light periodically.

Use of heat. Anantharamaiah et al. (1) stated that by heating the tubes for 2 min in a boiling water bath, the results would compare closely with the gravimetric procedure. Various temperature exposures were checked for color stabilization after the optimum intensity was achieved in the allotted time. These tests were checked against a non-heated reaction for comparison. Figure 3 shows that non-heated reactions continue to develop in color intensity in a lactic acid culture even after 1 hr of color development. This could be attributed to acetylmethylcarbinol entering into the reaction and giving the red color complex.

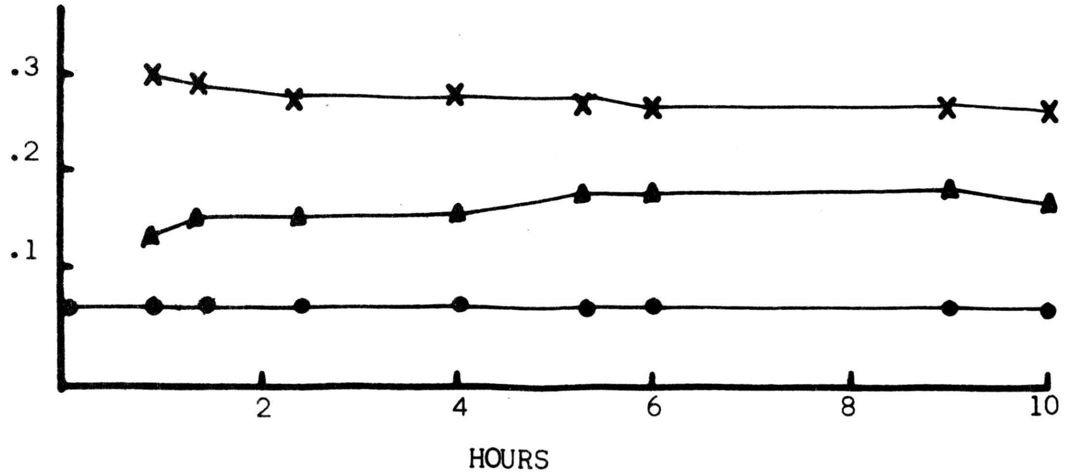
Comparing the points of origin on Figure 3, one can see the stabilizing effect that different times of heating have on the color stability in contrast to the unheated sample. The lower lines show the maximum color development at the initial reading after 20 min. The samples were heated for 2 min in a boiling water bath, cooled to room temperature and the optical density determined in the colorimeter. This method of treatment showed a decrease in color over a period of time for the standard concentration of diacetyl (7.0 ppm). However, it was not as pronounced as in the unheated sample. The downward slope of the line for the non-heated sample could also be attributed to the direct addition of a standard concentration of diacetyl. After 1 hr of color development the diacetyl content may have become exhausted. Loss of color takes place from the start of the color reaction, but at a much slower rate. At the end of 1 hr



7.0 PPM DIACETYL



CULTURE KF



SPREAD NO. 206

Figure 3. Comparison of heat treatment for different products assayed

▲ Non-heated, X Heated 5 minutes, ● Heated 2 minutes or more of reaction time, the rate of color production was less than the color degradation.

As the non-heated sample of the flavor culture KF and of the spread No 206 (experimental low fat dairy spread) are compared in Figure 3, a slight increase in optical density is noted. The increase may be attributed to additional acetylmethylcarbinol in the samples. If it were the acetylmethylcarbinol that was giving the enhanced color, it was developing faster than the original color was disappearing.

Heat has a stabilizing effect on the color reaction; however, the temperature and the length of time of heating varies from one type of sample to another. Comparing the three types of samples that were treated by heating for 5 min, 2 min and non-heating; the samples that were heated for 5 min tended to have the more stable color. However, the increased heating time on the sample from the spread increased the optical density readings. The other two samples (KF and 7.0 ppm diacetyl) had a lower optical density reading than the non-heated sample after they had been heated for 5 min. A heating time of 5 min appeared to be optimum for lactic acid cultures or acidified milk cultures; however, the sample of spread did not agree with these findings. Impurities in the filtrate from the spread sample could have entered into the reaction when the sample was heated for 5 min and gave a high optical density reading. A heating time of 2 min appeared to be optimum for the spread sample.

Assay of known amounts of diacetyl. A series of samples containing known amounts of diacetyl were assayed by the modified Voges-Proskauer reaction. Table 1 shows the reproducibility by this method. Actual values as determined by the Prill and Hammer method showed 8.8 ppm instead of 10 ppm as was calculated. Ten samples of each dilution were assayed by the modified Voges-Proskauer method to ascertain the range of the recovery. The average recovery for the samples that were assayed by the modified Voges-Proskauer method were compared with the value ascertained by the Prill and Hammer method. Average recovery values were calculated as shown in Table 3. Less than 5% error was incurred on the 6 ppm dilution; whereas, a smaller per cent error was incurred on all other dilutions. The range of extremes of the minimum and maximum values were calculated for each of the dilutions. The average value was divided into the lowest and highest value for each standard dilution. The values obtained from these calculations show a higher per cent error in the lower dilutions.

TABLE 1

Modified Voges-Proskauer method used to assay known amounts of diacetyl

	Standard diacetyl solutions (ppm)				
	10	8	6	4	2
	8.81	6.90	4.93	3.55	1.81
	8.52	7.90	5.11	3.80	1.81
	9.00	7.15	5.02	3.56	1.49
	9.06	7.08	4.98	3.56	1.60
	8.88	6.84	4.77	3.44	1.54
	8.91	7.04	5.02	3.49	1.75
	8.84	7.47	4.99	3.52	1.75
	8.73	6.93	5.43	3.35	1.86
	8.79	7.11	5.24	3.55	1.69
	8.84	7.02	5.14	3.51	1.76
Average Value (ppm)	8.84	7.14	5.06	3.53	1.71
Prill and Hammer Value (ppm)	8.80	7.04	5.28	3.52	1.76
Average Recovery (%)	100.45	101.42	95.83	100.28	97.16
Range of Extremes (%)	96.38 to 102.49	95.70 to 110.64	94.27 to 107.31	94.90 to 107.65	87.13 to 108.77

Assay of single strain cultures. The comparison in results between the modified Voges-Proskauer and the Prill and Hammer methods for single strain cultures are shown in Table 2.

TABLE 2

The ppm of diacetyl obtained when assaying non-acid producing single strain cultures by the Modified Voges-Proskauer and the Prill and Hammer method

Culture number	Modified Voges-Proskauer	Prill and Hammer
K-F*	4.90	7.15
K-F*	2.50	7.33
K-F*	7.30	8.60
LC-1	6.73	6.68
LC-2	7.99	6.20
LC-6	5.13	4.70
LC-7	9.28	8.38
LC-9	10.14	8.38
K-F*	9.21	8.63

*All K-F cultures were the same non-acid producing single strain culture, however, they were assayed on different days.

The single strain non-acid producing cultures (K-F, LC-1, LC-2, LC-6, LC-7 and LC-9) had high diacetyl values when assayed by the modified Voges-Proskauer and the Prill and Hammer methods. The single strain cultures were grown for 48 hr before the pH was lowered to 4.4 with sterile 15% citric acid. After 18 hr of incubation in the citric acid acidified culture, the ppm of diacetyl was assayed by the modified Voges-Proskauer and the Prill and Hammer methods.

Assay of multiple strain lactic cultures. Multiple strain lactic acid cultures were also assayed by the modified Voges-Proskauer and the Prill and Hammer methods. The comparison is shown in Table 3.

TABLE 3

The ppm of diacetyl obtained when assaying multiple strain lactic acid cultures by the Modified Voges-Proskauer and the Prill and Hammer Method

Culture number	Modified Voges-Proskauer	Prill and Hammer
M-9	11.20	3.35
H-5*	.88	.65
H-5*	4.40	1.38
H-5*	5.10	1.37
K-5	13.20	2.76

*All H-5 cultures are the same multiple strain lactic-acid cultures, but were assayed on different days.

Lactic acid cultures (M-9, H-5 and K-5) had lower values of diacetyl in the Prill and Hammer method; although the same samples had high values of diacetyl when assayed by the modified Voges-Proskauer method. The higher results obtained by the modified Voges-Proskauer could be attributed to acetylmethylcarbinol entering into the reaction and causing enhanced red color complex.

SUMMARY AND CONCLUSIONS

The Prill and Hammer method of determining diacetyl has been used quite extensively for the last 30 yr. Even though a low degree of deviation was observed in the results, it was a slow, meticulous test. Various tests have been introduced that shortened the length of time and were simpler to run; however none of these tests became very prominent. The Voges-Proskauer test has been checked as a quantitative measurement for diacetyl. Modifications by O'Meara (15,16) and Barritt (2) increased the speed and the color intensity of the Voges-Proskauer reaction.

Anantharamaiah _et_ _al_. (1) introduced a method of detecting diacetyl with the Voges-Proskauer test. Acetylmethylcarbinol required 1 hr for maximum reaction, whereas diacetyl required 10 min. They claimed that diacetyl estimated in this manner would compare closely with the gravimetric procedure. The modified Voges-Proskauer method introduced by these workers was studied. Some of the procedures that they used were not successful in this investigation. When trichloracetic acid was used to deproteinize milk cultures, a flocculent precipitate masked the color reaction and interfered with the optical density readings. A blue color was also present when the trichloracetic acid filtrate was used in the reaction. The blue color was more prevalent in the tubes containing 4 ppm diacetyl or less. It was present in the tubes with the higher concentrations of diacetyl, however, the greater intensity of red color was not masked to any great extent. Erroneous readings were obtained because of the different optical density produced by the blue color complex.

A wavelength of 540 mμ was found to satisfy the conditions of the test. Anantharamaiah _et_ _al_. (1) used a wavelength of 490 mμ, and stated that this lower wavelength showed the least interference from impurities. With the elimination of trichloracetic acid from the test, a clear filtrate was obtained which made it possible to use the higher wavelength.

Creatine was used as the guanido donor instead of arginine in the development of the red pigment. When arginine was used, the intensity of the color was reduced. The lighter shade of red may be attributed to the longer chain of the arginine molecule in comparison to the creatine molecule. The lighter shade of red made optical density readings less accurate at the lower concentrations. Arginine also is part of the casein molecule, which when subjected to acid or heat hydrolysis, can yield arginine. It can enter into the reaction and give erroneous results. Creatine is present in milk, but in such low quantities that it has no effect on the reaction.

Different diluting agents were used to determine optimum color intensity. Methyl, ethyl, n-propyl and n-butyl alcohols were used as the diluting agents; however, none of the alcohols met all the desired requirements. A precipitate was formed by all of the alcohols. Double distilled water was the best diluting agent. It failed to produce as bright a red color as ethyl alcohol, but it did not cause any precipitation.

Fresh α-naphthol should be used for the reaction or erroneous results may be obtained. It is not necessary that the α-naphthol be distilled before use; although a fresh supply should be kept in a cool, dark storage area. This retards oxidation of the molecule thus minimizing the dark brown color.

Heating the tubes prior to reading in the colorimeter tended to have a stabilizing effect on the color. A comparison of different lengths of heating time in a boiling water bath were studied. The color was stabilized more in the heated samples than in a non-heated samples. The non-heated samples showed an increase in optical density after the initial reading. This increase was evident in all samples for at least the first hr of color development. However, in the sample with a known amount of added diacetyl, color development reached a plateau in less time. The color development continued to increase in the non-heated sample of a lactic acid culture and the spread. This lengthened time could be attributed to acetylmethylcarbinol entering into the reaction.

Reproducibility of known amounts of diacetyl when assayed by the modified Voges-Proskauer method showed relatively close agreement with the Prill and Hammer method. When assaying single strain cultures, the agreement between the two different methods was also comparable. However, when multiple strain lactic acid cultures were assayed, the modified Voges-Proskauer values were higher than the Prill and Hammer values. These higher values could be attributed to acetylmethylcarbinol entering into the Voges-Proskauer reaction. The overall agreement between the two different methods of diacetyl analysis showed that the modified Voges-Proskauer method could be used as an approximate evaluation or as a screening test.

The modified Voges-Proskauer test has some undesirable aspects that need additional work. Simplicity and a short reaction time are its highest attributes. Additional work will have to be done to make it a more accurate method of detecting diacetyl. From the moment the reaction begins, a red color complex is being formed, however it is being destroyed at a slower rate. Acetylmethylcarbinol can enter the reaction after oxidation, the same as diacetyl. With this in mind, the amount of red color complex is not only proportional to the amount of diacetyl but is also proportional to the acetylmethylcarbinol in the culture.

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