XXXII. A COLORIMETRIC REACTION FOR THE QUANTITATIVE ESTIMATION OF NICOTINIC ACID

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Now that it is known that nicotinamide is a component of cozymase, and that nicotinic acid possesses a curative effect on pellagra, a method by which this substance could be determined qualitatively and quantitatively, and which would be suitable for serial work, would be valuable.

Methods of analysis have been published previously by Swaminathan [1938], Karrer & Keller [1938], Vilter et al. [1938] and quite recently by Shaw & Macdonald [1938]. None of these methods is based on a specific reaction with nicotinic acid; they are founded on a colorimetric reaction of the pyridine ring. Swaminathan's and Shaw & Macdonald's methods are based on the observation made by König [1904], that pyridine with cyanogen bromide and a primary or secondary aromatic amine develops a colour, which, according to the amine employed, varies from yellow to violet. The chemical reactions leading to colour formation have not yet been fully elucidated. Kulikow & Kresdowosdwigenskaja [1930] worked out a method based on this for determining small amounts of pyridine using aniline. Karrer & Keller [1938] and Vilter et al. [1938] based their work on the observation of Vongerichten [1899] who found that a colour reaction ensues when pyridine reacts with 2:4-dinitrochlorobenzene and alkali hydroxide.

Up to the present none of these methods has proved quite satisfactory. Swaminathan [1938], who as mentioned used aniline, extracted the resulting colour by shaking with isoamyl alcohol. In our hands this method has yielded unsatisfactory results; the colour developed is inconstant and it is difficult to obtain pyridine-free isoamyl alcohol.

We have not had the opportunity to test Shaw & Macdonald's [1938] method of analysis, as this appeared after we had completed the experimental part of this work. The authors, however, state that the intensity of the colour is not constant at any time, which renders their method unsuitable at any rate for serial analyses. The methods described by Karrer & Keller [1938], and by Vilter et al. [1938], appear to be laborious and tedious, and the authors mention that the colour obtained is very unstable.

In the present work, an account is given of a colour reaction based on König's principle. It is rapid to carry out, is performed in aqueous solution and gives a constant colour.

As colour-producing amine we have tried Na sulphanilate, Rodinal (p-amino-phenol) and sulphanilate (p-aminobenzenesulphonamide), which however gave inconstant results, whereas we found that metol (p-methylaminophenol sulphate) with nicotinic acid and CNBr in aqueous solution yields a clear yellow colour which, under the conditions used by us, is perfectly constant, very stable and of an intensity directly proportional to the amount of nicotinic acid.
I. ESTIMATION OF NICOTINIC ACID

A. Determination of nicotinic acid in colourless aqueous solution

**Technique**

A measured amount (up to 9 ml.) of the aqueous solution of nicotinic acid to be analysed (containing from 0-005 to 0-25 mg. nicotinic acid) is run into a graduated 20 ml. flask. After 5 min. heating on a water bath at 75–80°, 1 ml. 4 % aqueous CNBr is added. The mixture is placed on the water bath for 5 min. and then cooled under the tap to room temperature. 10 ml. saturated aqueous metol (about 5 %), and distilled water to make up the volume to 20 ml., are added. After standing 1 hr. at room temperature excluded from light, the strength of the colour developed is read off with a Pulfrich photometer (filter S. 43), with a blank solution containing the same amounts of CNBr and metol plus distilled water to make the volume 20 ml. in the other cell.

**A. The reagents**

1. *Cyanogen bromide.* We have used CNBr supplied by Fraenkel & Landau, Berlin-Oberschöneweise. A 4 % aqueous solution is employed, and it must be prepared on the day of use.

A solution of CNBr prepared as described by Kulikow & Kreslovosdwienskaja [1930] may also be used. To a saturated aqueous solution of Br, KCN is added just to decoloration. This solution contains about 5 % CNBr.

2. *Metol (p-methylaminophenol sulphate)*; \( \text{CH}_4\text{NHC}_6\text{H}_4\text{OH}_2, \text{H}_2\text{SO}_4 \). The metol employed is the usual product supplied by Agfa. An aqueous solution, saturated at room temperature, i.e. c. 5 %, is used. Thorough shaking is necessary as the metol dissolves rather slowly and the solution must not be heated. It is protected from light and must be used within 2–3 hr. of preparation. Either on heating or on further standing the solution turns brownish-violet.

**B. The analysis**

1. The reaction between nicotinic acid and CNBr

   (a) **Influence of temperature.** At room temperature the reaction between nicotinic acid and CNBr proceeds very slowly. At 75–80° the reaction takes place so quickly as to be completed in about 3 min. If the mixture is heated to this temperature for more than 20 min. the compound first formed slowly disintegrates.

   (b) **The amount of CNBr.** At least 30 mg. CNBr must be used for amounts of nicotinic acid up to 0.25 mg. Larger quantities (up to 80 mg.) do not change the extinction constant; but smaller quantities give too low an extinction value.

2. The reaction with metol of the compound formed by nicotinic acid and CNBr

   (a) **Amount of metol.** The extinction constant increases with the concentration of metol in the solution until a certain point is reached. For metol quantities of 500–650 mg. in a volume of 20 ml. (i.e. from 10 to 13 ml. 5 % solution) the extinction is constant (see Fig. 1). The quantity of metol determined in this way appears rather large in relation to the amount of nicotinic acid. On recrystallization of the metol exactly the same results were obtained; thus the colour reaction can hardly be ascribed to impurities in the reagent. The final intensity of the colour depends on the metol concentration and not on the absolute quantity of metol: with a certain amount of nicotinic acid plus 250 mg.
metol diluted to a total volume of 10 ml. the colour intensity will be exactly twice that resulting from the same amount of nicotinic acid plus 500 mg. metol in a volume of 20 ml.

(b) *Time of reaction with metol.* On addition of metol a faint yellow coloration appears immediately and quickly intensifies. The intensity of the colour, however, first reaches its maximum after \( \frac{1}{4} \) hr.

(c) *Constancy of the colour developed.* At room temperature with protection from light the colour of the solution remains unchanged after 72 hr.

(d) *The effect of light.* If, after metol has been added, the mixture is kept in full daylight, the colour developed is fainter than if the solution has been kept in darkness; continuous exposure will result in gradual fading. However, the sensitiveness to light is not so great that the colour fades appreciably in diffuse daylight in 5–10 min.

(e) *The temperature.* No change occurs in the colour produced if the solution, after adding metol, is kept for 1 hr. at temperatures ranging from 5 to 30°. At 75–80° the colour developed is considerably fainter, beginning as a clear yellow it subsequently, after 20–30 min., turns more brownish-red.

3. *Photometric determination*

(a) *Colour.* The colour developed is a clear yellow. The extinction corresponding to the various filters of the Pulfrich photometer are approximately (0·1 mg. nicotinic acid in 1 ml. layer): S. 43, 0·89; S. 45, 0·48; S. 47, 0·26; S. 50, 0·05; S. 53–S. 75, 0·00.

(b) *Measurement.* With cells of 2·5–50 mm., it is possible to determine nicotinic acid in quantities ranging from 0·005 to 0·25 mg. Larger quantities yield an extinction which is too high to be measurable in a layer of 2·5 mm. In case the colour is too intense, diluting the solution with water before measurement is without avail, as this lowers the concentration of metol, thus changing the extinction constant. A fresh analysis must be performed on a more diluted solution of the sample to be examined.

(c) *Blank.* The blank is made with distilled water in place of the nicotinic acid solution. Blanks give a very pale pink colour. It has been observed that
CNBr preserves metol from changing to the coloured substances mentioned above; even after standing for several days in full daylight the colour of the blank does not change perceptibly.

Using the filter (S. 43) employed in the analysis in a layer of 1 cm. the blank analysis with distilled water gives an extinction which, reckoned from the moment of adding metol to 24 hr. later, gradually increases from 0.02 to 0.04.

4. Determination of the extinction curve

With known amounts of nicotinic acid the extinctions given in Fig. 2 have been found. Within the limits of the quantities measured the colour developed is directly proportional to the amount of nicotinic acid.

Under the conditions mentioned, we have not found any variation in the extinction constant; nevertheless, as a control in each series of analyses, we have determined the value of a standard solution with known content of nicotinic acid (0.1 mg.). This has, in addition, served as a control on the reagents.

For working out the standard analysis 1 ml. of a 1:10,000 solution of nicotinic acid may suitably be used. In this feeble concentration the nicotinic acid is stable for a few days only. In a concentration of 1:1000, and in a brown glass receptacle, nicotinic acid is stable for several months at least.

5. The sensitiveness and accuracy of the method

As mentioned, quantities of nicotinic acid as small as 0.005 mg. may be determined with great accuracy.

It is difficult to give the percentage error. After numerous tests we believe that when pure aqueous solutions of nicotinic acid are used the error of the method is derived chiefly from the error in the photometric readings.

The method of analysis described has been worked out with pure aqueous solutions of nicotinic acid. An equiv. wt. of 122.4 (calculated 123.05) was found for the nicotinic acid used (J. D. Riedel-E. de Haën, A. G.); m.p. 232°, as stated in the literature.
The colour reaction does not tolerate free strong acid or base. In the presence of acetate ions a red-brown colour develops. Sulphate (in higher concentration than it occurs in metol) and nitrate ions cause no change in the tint, but weaken the intensity of the colour.

NaCl, NH₄Cl and KH₂PO₄ cause no change in either the tint or intensity of the colour.

If the reaction occurs in presence of acetone or alcohol, the reading is lower and inconstant.

**Nicotinamide**

If nicotinamide is examined by the process described above, the same results are not obtained as for equivalent amounts of nicotinic acid. The same clear yellow colour is produced, but it is usually considerably stronger, and the results are not reproducible. Therefore if nicotinic acid occurs partly or wholly as amide, it must be hydrolysed before the analysis.

### II. Determination of Nicotinic Acid in Organic Material

In biological material, nicotinic acid occurs—at any rate to a large extent—in the form of the amide as a component of cozymase or Warburg's coferment; prior to determination it must, therefore, be liberated and the nicotinamide hydrolysed. We have found that this can be accomplished by heating with NaOH: as it appeared that the same result was obtained by heating in a boiling water bath for ¹⁄₂ hr. as by heating in the autoclave for periods up to 2 hr. at 120°, followed by heating with 2N HCl (Table I), the first procedure was adopted.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dried yeast + 50 mg. nicotinic acid (as amide) per 100 g. Total nicotinic acid found mg. %</th>
<th>Added nicotinamide found (as nicotinic acid) %</th>
</tr>
</thead>
<tbody>
<tr>
<td>On boiling water bath for:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 min.</td>
<td>53-2</td>
<td>—</td>
</tr>
<tr>
<td>60 min.</td>
<td>54-2</td>
<td>105-7</td>
</tr>
<tr>
<td>90 min.</td>
<td>53-4</td>
<td>—</td>
</tr>
<tr>
<td>120 min.</td>
<td>53-2</td>
<td>—</td>
</tr>
<tr>
<td>Heating in an autoclave at 120° for:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 min.</td>
<td>53-2</td>
<td>102-5</td>
</tr>
<tr>
<td>60 min.</td>
<td>53-0</td>
<td>103-5</td>
</tr>
<tr>
<td>120 min.</td>
<td>53-2</td>
<td>97-6</td>
</tr>
</tbody>
</table>

Nicotinic acid added to the samples was in every case recovered quantitatively by these methods. This process results in a highly coloured homogeneous mass, which must be rendered quite colourless before it is suitable for colorimetric determination.

The removal of the colour by adsorption with charcoal or Fuller's earth at different reactions was unsuccessful, as the nicotinic acid is adsorbed with the coloured substances, and cannot be entirely eluted separately. As even filter paper adsorbs nicotinic acid, filtering must not take place at any stage of the analysis. Nor was precipitation of the coloured substances with heavy metal salts and subsequent removal of the metal ions with H₂S satisfactory, while HgCl₂ and Ag₂SO₄ did not precipitate the coloured substances. Pb acetate and Hg(NO₃)₂ cannot be used as the anions of these salts interfere with the colour.
ESTIMATION OF NICOTINIC ACID

reaction. The best result, although still not satisfactory, was obtained by precipitation with CuCl₂. Evaporation to dryness and subsequent extraction with benzene and acetone were discarded, as neither the preformed nor the added nicotinic acid could be extracted quantitatively after drying.

However, it was found that the coloured substances could be precipitated from an aqueous phase by a large excess of acetone, in which nicotinic acid is relatively easily soluble. We have employed this procedure to remove the colour. Based on these experiments, we have worked out a method of analysis. As an example, the technique used in examining dry yeast will be described in the following.

Technique

10 ml. 2 N NaOH are run into a 20 ml. graduated flask without moistening its neck, and 5 g. of dry yeast are added (the order must not be reversed as the yeast will then collect in lumps which will merely be attacked superficially). The neck of the flask is closed with a wad of non-absorbent cotton wool. After shaking, the flask is placed on a boiling water bath for 30 min.; the yeast will then be fully dissolved. After cooling, concentrated 36 % HCl (1·8 ml.) is added drop by drop, and the mixture thoroughly shaken. The reaction will now have become slightly acid (pH=4-6). The solution is cooled to about 20°, and distilled water is added to make up the volume to 20 ml.

The contents are thoroughly mixed and allowed to stand for several minutes. Part is then centrifuged in a 15 ml. centrifuge tube. A voluminous sediment (amounting to about one-half to one-third of the total) is obtained; the upper layer consists of a slightly turbid dark brown liquid.

Exactly 1 ml. of the centrifugate (corresponding to 250 mg. yeast) is placed in a second centrifuge tube, which is well shaken while exactly 9 ml. acetone are added slowly from a burette. The tube is carefully closed with a rubber stopper, and after vigorous shaking, it is centrifuged for 3-4 min. The contents will then have separated into two layers—a very small (about 0·3 ml.) intensely coloured very viscid aqueous phase, and a clear practically colourless layer of aqueous acetone.

3 ml. (corresponding to 75 mg. yeast) of the acetone layer and 3 ml. distilled water are then mixed in a round-bottomed flask and the acetone evaporated with a water vacuum pump, without other heating than the warmth of the hand. After evaporation of the acetone, the contents are quantitatively transferred with the aid of N/15 KH₂PO₄ (which ensures that the reaction does not become too acid) to a graduated flask of 20 ml. capacity. The volume must not exceed 9 ml.

The colour reaction described above is carried out on the practically clear colourless solution thus obtained and, with the aid of the coefficient of extinction controlled by the standard solution (0·1 mg. of nicotinic acid), the amount of nicotinic acid contained in the sample of yeast is determined.

Comments

After treatment with NaOH, the yeast becomes a homogeneous mass; a voluminous precipitate appears on cooling and particularly on neutralizing with hydrochloric acid. The mixture is cleared by centrifuging and aliquot parts are employed for the extraction with acetone. That this is allowable is shown by the fact that the intensity of the colour produced is the same whether a part of the clear centrifugate or a part of the total mixture is used. However, it is
advisable to remove the precipitate by centrifuging; otherwise, on transfer-
ence of the acetone extract to water, a turbidity will appear, which—although
diminishing considerably during the colour reaction—nevertheless complicates
the photometric determination. The error in the readings may amount to 5% at most; this error is avoided by removing the precipitate.

With regard to the acetone extract the following point must be mentioned:
if too large an excess of acetone is used, a fairly solid resinous precipitate
is formed, containing a relatively large part of the nicotinic acid (about 30%).
On the other hand, too small an amount of acetone will not completely precipitate
the coloured substances.

The principle to be observed in extraction is to use exactly that quantity of
acetone which gives a sufficiently colourless and clear solution without leaving a
precipitate that is too solid.

In the case of yeast this is achieved by using the quantities described. The
precipitate amounts to about 0.3 ml., and consists partly of solid flakes, and
partly of a very viscid brown liquid. Analysis of the precipitate (dissolved in
0.7 ml. distilled water and again extracted with acetone) has shown that the
concentration of nicotinic acid is the same as in the acetone phase.

The transference of the acetone extract to water is rendered necessary by
the fact that the presence of even small quantities of acetone during the process
of heating with CNBr will result in lower values.

It is essential to evaporate the acetone at low temperature; high temperature,
e.g. heating on a boiling water bath, will cause some of the nicotinic acid to
volatileize with the acetone. Control analysis with known quantities have shown
that the nicotinic acid is recovered quantitatively when the acetone is evaporated
in vacuum with no other heating than the warmth of the hand. By evaporation
on a boiling water bath of an aqueous solution of nicotinic acid (containing
about 0.1 mg. of nicotinic acid) the loss is quite large; it is least when the solution
contains NaOH, but very considerable if the solution is made alkaline with NH₃
or acid with a mineral acid. Presence of acetone during evaporation increases
the loss of nicotinic acid.

The analysis of various kinds of yeast has given the results shown in Table II.

<table>
<thead>
<tr>
<th>Yeast Type</th>
<th>Nicotinic Acid in Dry Matter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tuborg</td>
<td>140 mg. %</td>
</tr>
<tr>
<td>168 Surface-yeast</td>
<td>45.5</td>
</tr>
<tr>
<td>113 Bottom-yeast</td>
<td>44.6</td>
</tr>
<tr>
<td>173 Bottom-yeast</td>
<td>36.2</td>
</tr>
<tr>
<td>175 Bottom-yeast</td>
<td>33.9</td>
</tr>
<tr>
<td>7 Wine-yeast</td>
<td>36.7</td>
</tr>
<tr>
<td>11 Wine-yeast</td>
<td>17.7</td>
</tr>
<tr>
<td>Faex medicinalis</td>
<td>I 61.2</td>
</tr>
<tr>
<td>II 57.2</td>
<td></td>
</tr>
<tr>
<td>III 55.9</td>
<td></td>
</tr>
<tr>
<td>Compressed baker’s yeast</td>
<td>25.7</td>
</tr>
</tbody>
</table>

**Summary**

An exact colorimetric method of analysis for determining nicotinic acid in
aqueous solution has been elaborated.

The method depends on the addition of cyanogen bromide at 70–80° and the
production of a colour with metol (p-methylaminophenol sulphate) at room
temperature.
ESTIMATION OF NICOTINIC ACID

The method has been employed to analyse samples of yeast. From 16 to 61 mg. of nicotinic acid % dry wt. have been found in the various types of yeast.

We wish to thank the Tuborg Breweries for submitting the yeast samples.

REFERENCES

Shaw & Macdonald (1938). Quart. J. Pharm. 11, 380.