Determination of Amino Nitrogen, Pyrrolidone Carboxylic Acid Nitrogen, and Total Nitrogen With Ninhydrin

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Introduction

For a general picture of type impurities in beet juices, it is important to know the total amino acid constitution and so to have a rapid and accurate method for this determination.

Ninhydrin (1,2,3 triketohydrindene) is probably the most useful general reagent for amino acids. But for colorimetric procedures, it long had the drawback of giving different color depths for different amino acids and was not considered suitable for total amino acid determinations (10)[2] except by the unwieldly measurement of CO_2 released in the reaction (11).
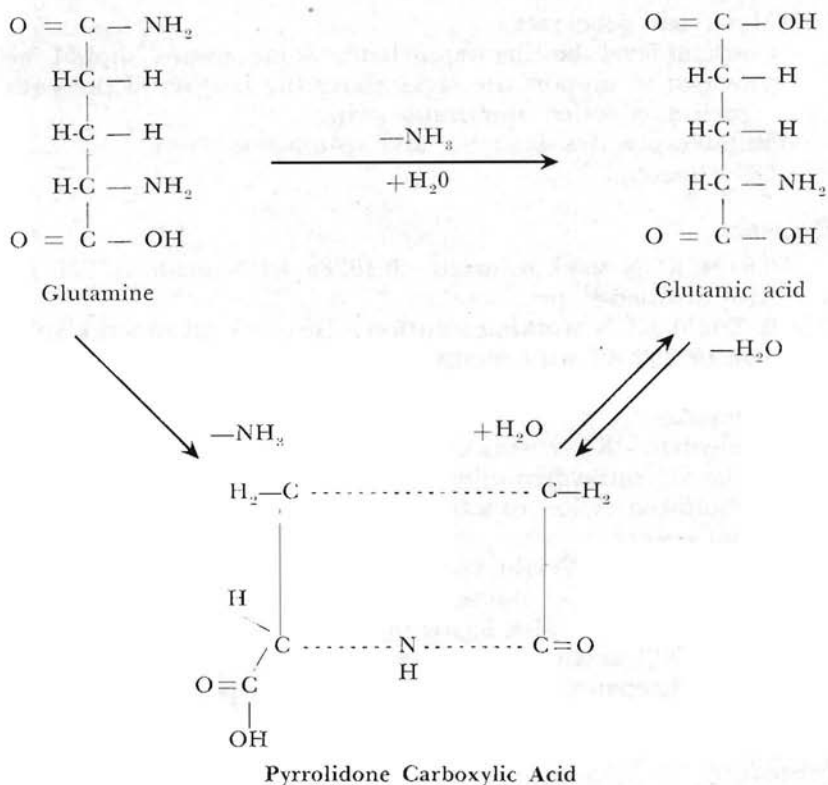
However, in the early 1950's, several workers had developed the technique to a point that gave 100% color development from the majority of common amino acids. Troll and Cannan (13) used a phenol-alcohol-pyridine system with a combination of ninhydrin and its reduction product, hydrindantin. Moore and Stein (8) improved their previous method, wherein stannous chloride was used to form reduced ninhydrin, by adding hydrindantin itself with ninhydrin in a methyl cellosolve system buffered with 4N sodium acetate at pH 5.5. Yemm and Cocking (15) used KCN to form hydrindantin directly in a methyl cellosolve system containing ninhydrin and buffered with 0.2 M sodium citrate at pH 5.0.

Although these methods are suitable for total amino nitrogen evaluations in most circumstances, beet juices in processing present an additional problem. Here large amounts of pyrrolidone carboxylic acid (PCA), a compound insensitive to ninhydrin, are formed at the expense of glutamine. The ammonia released in this conversion does react to a significant extent in the ninhydrin systems mentioned.

PCA probably derives entirely from glutamine (4,12,6,2); so that the amount of PCA present in the juices directly reflects the amount of glutamine present in the original beet. So, for all practical purposes, PCA must be considered in the amino acid spectrum of factory juices:

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[2] Numbers in parentheses refer to literature cited.

$$
\begin{array}{ccc}
O = C - NH_2 & & O = C - OH \\
\mid & & \mid \\
H\text{-}C - H & & H\text{-}C - H \\
\mid & & \mid \\
H\text{-}C - H & \xrightarrow[\;+H_2O\;]{-NH_3} & H\text{-}C - H \\
\mid & & \mid \\
H\text{-}C - NH_2 & & H\text{-}C - NH_2 \\
\mid & & \mid \\
O = C - OH & & O = C - OH \\
\text{Glutamine} & & \text{Glutamic acid}
\end{array}
$$

$-H_2O$

$-NH_3$ $+H_2O$

$$
\begin{array}{c}
H_2\text{—C} \cdots\cdots\cdots\cdots \text{C—}H_2 \\
\mid \qquad\qquad\qquad\qquad\quad \mid \\
H \\
\searrow C \cdots\cdots N \cdots\cdots C = O \\
O = C \qquad\quad H \\
\mid \\
OH
\end{array}
$$

Pyrrolidone Carboxylic Acid

The method described here is based on the procedure of Yemm and Cocking (15). The important difference is in the buffer system and the way it is used.

To determine total amino nitrogen, a two component acetate buffer is used. For the simultaneous determination of PCA, the caustic fraction of the buffer is used to accomplish rapid and complete hydolysis of PCA to glutamic acid; the acid fraction is then added; the sample is made to proper volume; and the ninhydrin reaction is carried out and colorimetric evaluation made.

Ammonium nitrogen was noted to give a constant response to ninhydrin. This led to development of a technique for Total Nitrogen that compares in accuracy to the Kjeldahl method, but requires no distillation step.

Methods
Determination of Amino Nitrogen
Equipment

Pyrex test tubes, 20 × 150 mm, calibrated at the 2-1/2 ml level.

#2 rubber stoppers

Metal test tube racks

Constant-level boiling-water bath. Some means should be provided to support the racks above tne bottom of the bath —such as a coiled aluminum strip.

Suitable pipettes, burettes, and volumetric flasks.

Colorimeter

Reagents

0.01M KCN stock solution - 0.1628g KCN made to 250 ml with distilled water.

0.0002M KCN working solution - Dilute 5 ml of stock solution to 250 ml with methyl cellosolve (2 - methoxyethanol)

5% ninhydrin solution - Weight/volume solution in methyl cellosolve.

Ninhydrin - KCN working solution (optional) - Mix 50 ml of the 5% nnihydrin solution with 250 ml of the KCN working solution. Allow to stand a few hours before using. Stable about a week.

20% NaOH - Weight/volume solution in distilled water.

20% acetic acid - volume/volume solution in distilled water.

Buffer solution - Mix 1 part of the 20% NaOH with 2 parts of the 20% acetic acid. pH should be 5.0 - 5.05.

50% isopropanol - Equal volumes of isopropanol and distilled water.

Procedure

To establish a standard curve, prepare a series of dilutions of pure glutamic acid to contain 0 to 0.01 mg of amino N per ml. These are run through the procedure described below for juice samples. A perfectly straight line results when mg of N are plotted against % transmittance on 2-cycle semi-log graph paper.

Juice samples are diluted to contain between 2 and 10 gamma of amino N per ml. Readings are usually within this optimum range with dilutions of 1 to 50-100 on juices through 2nd carbonation and thin, 1 to 100-200 on thick juices and 1 to 500-1000 on molasses.

Pipette 1 ml of diluted sample (or standard) to test tube, add 1-1/2 ml of buffer and mix by shaking.

Now add 1.2 ml of ninhydrin-KCN working solution (or 1.0 ml of KCN and 0.2 ml of ninhydrin may be added separately). Mix thoroughly and stopper the tube loosely to prevent undue evaporation.

React the mixture by placing the racked test tube in the boiling water bath for 15 minutes. Then cool in running tap water for about 5 minutes, dilute with 10 ml of 50% isopropanol, and mix.

Read % transmittance on the colorimeter at 570 millimicrons, using distilled water for 100% transmittance. Translate the amount of amino nitrogen from the standard curve.

Under the conditions of this procedure, ammonium nitrogen gives about 45% as much color as does amino nitrogen. Consequently, if the sample contains much ammonium nitrogen, its amount should be determined and the proper correction made. We determine ammonia by a 10 minute distillation, under vacuum at 55-60° C, from a pH 10.0 borate buffer (14).

To illustrate: a diluted molasses sample contained 1.002 mg of dry matter per ml. One ml gave 32.8% transmittance on the B and L "Spectronic 20". This reads as .0037 mg of amino N from the standard curve - or 0.369% amino N on dry matter.

The ammonium N had been determined as 0.006% on dry matter. Therefore, it contributed to the amino N determination in the amount of $0.006 \times .45$ or 0.003%.

The actual amino N content was, therefore, $0.369 - .003 = 0.366\%$ on dry matter.

Obviously ammonium nitrogen is not a significant factor in molasses, or even in thick juice. In all other juices, though— from diffusion juice through thin—it is quite significant and, unless accounted for, can lead to 20 to 30% error in the amino nitrogen determination.

Determination of Total Amino plus PCA Nitrogen

Equipment
    As for Amino N

Reagents
    40% NaOH — Weight/volume
    Others as for Amino N

Procedure
    From a suitably graduated pipette or burette, add 0.25 ml of 40% NaOH to the test tube containing 1 ml of sample. Place the open tube in a test tube rack in the boiling-water bath for at least 20 minutes. Within this time any ammonia is expelled and PCA is quantitatively converted to glutamic acid.

Cool the sample, add 1 ml of 20% acetic acid, and adjust the volume to the 2-1/2 ml mark with distilled water. At this point the tube contains 1 ml of sample and 1-1/2 ml of buffer exactly as used in the amino nitrogen determination.

Proceed exactly as for amino nitrogen: add 1.2 ml of ninhydrin-KCN; mix; stopper loosely; react for 15 minutes in the boiling-water bath; cool; dilute with 10 ml of 50% isopropanol; read at 570 millimicrons.

The color intensity now reflects the amount of original amino nitrogen plus the amount of PCA nitrogen.

For example, the molasses sample cited previously gave a reading of 11.7% transmittance after hydrolysis. From the standard curve, this translates to .00744 mg of total amino nitrogen or 0.743% on dry matter.

Since the original amino nitrogen content was 0.366%, it is apparent, by subtraction, that 0.377% was present as PCA nitrogen.

Determination of Total Nitrogen

Under the conditions of the test just described, ammonium N was observed to give about 45% as much color as an equivalent amount of amino N. The reaction is not completed within the 15 minute time interval, but progresses as time of heating is extended. However, the constancy of the reaction was the clue for possible adjustment of reagents and conditions to allow determination of Total Nitrogen.

Okada and Hanafusi (9) developed an ultramicro-determination of total organic N with ninhydrin. Their method requires the usual digestion and distillation, with ammonia being determined on the distillate.

The procedure devised here requires no distillation. It has given consistently excellent agreement with duplicatd Kjeldahl determinations on pure nitrogenous compounds, molasses and beet juices, and on various feedstuffs. In 1958, seventy molasses samples of the previous campaign were divided for the ninhydrin analysis to be used at this laboratory and the Kjeldahl analysis to be made at another laboratory. Results agreed so well, that we now use the ninhydrin procedure for all total N determinations.

After the digestion step the procedure is basically the same as for the determination of amino N, and can be done on the same "production line" basis.

Equipment

> Digestion facilities; digestion flasks of 100 ml capacity or large pyrex test tubes and 100 ml Kohlrausch flasks.
>
> Other materials as for Amino N determination.

Reagents

> Conc. H_2SO_4; nitrogen-free Na_2SO_4; catalytic mixture of $100g$ $K_2SO_4 + 10g$ $HgO + 5g$ selenium
>
> > 0.1% Methyl red indicator in ethanol
> >
> > Dilute $H_2SO_4 = 0.2$　　0.5 N
> >
> > 20% NaOH
> >
> > 10% ninhydrin in methyl cellosolve
> >
> > 0.0002M KCN in methyl cellosolve (as for amino N)

Ninhydrin - KCN working solution, Mix one volume of ninhydrin with 2 volumes of .0002M KCN.

Buffer (as for amino N) 1 part 20% NaOH - 2 parts 20% 20% acetic acid.

50% isopropanol (as for Amino N)

Procedure

Digestion and preparation of sample

Transfer sample, containing 1 mg N or less, to digestion flask.

Add about 500 mg Na_2SO_4, 20 mg of the digestion mixture, and 1-2 ml of H_2SO_4.

Place flask over reduced heat until any H_2O is boiled off and foaming subsides, then boil rapidly to a water white solution. (about 20 minutes.)

Cool flask sufficiently to add 60-70 ml of H_2O. Then add 2 to 3 drops of methyl red and neutralize as follows: Add 20% NaOH until the red color is just discharged. Then add dilute H_2SO_4 until the solution is just acid to methyl red. A little excess is of no consequence, but a large excess should be avoided.

Cool to room temperature and make to the 100 ml mark.

Determination of N

Transfer 1 ml of the neutralized digest to test tube. Add 1-$\frac{1}{2}$ ml of buffer and mix, then add 1-$\frac{1}{2}$ ml of the ninhydrin KCN solution and mix.

Stopper loosely and place in the test tube rack in the boiling water bath for 15 minutes.

Cool, add 10 ml of 50% isopropanol, and mix.

Read transmittance at 570 millimicrons and translate the reading to mg of N from a standard curve.

To establish the standard curve, any pure N-containing compound—such as recrystallized hippuric acid— may be run through the digestion step. However, $(NH_4)_2SO_4$ is more easily used and the digestion step can be omitted if desired. Transmittance from dilutions containing 0 to .01 mg N per ml plot as a straight line on 2-cycle semi-log graph paper.

Discussion

These procedures are carried out on several samples simultaneously. For example, our racks each hold 16 test tubes which allow eight simultaneous, duplicated, determinations. While one rack of samples is reacting, another is being prepared. The reacted and cooled samples may be held several hours without color deterioration. Even after dilution with isopropanol, the color is stable two or three hours.

The extreme sensitivity of the reagent demands the use of well cleaned glassware. It is found that occasional boiling in alconox solution is very satisfactory.

The ninhydrin and KCN are mixed as a time-saving measure when many determinations are being made. The mixture is less stable than the individual solutions, and will begin showing a weaker reaction after about a week. It may still be used, provided standards are re-run and readings taken from the new curve. However, once started, the deterioration progresses rapidly and a new solution must be made to restore readings to the original curve.

Occasional standards should be run, but deviations are minor so long as the same cellosolve is being used in the re agents. Since a little color develops from impurities in the cellosolve, the curve must be rechecked when a different batch of this reagent is used. It is customary to re-distill each batch of cellosolve to minimize this impurity interference.

Standard amino acid solutions must be watched carefully. Stock solutions containing 0.1 mg N per ml seem to retain their strength for several days, but standard solutions containing 0.01 mg N per mg, or less, invariably begin showing a weaker reaction after two or three days, even when kept refrigerated. The cause of this deterioration is not known, but it has been encountered by other workers (5).

Most amino acids, based on equivalent amino nitrogen content, fall exactly on the standard curve for glutamic. A few amino acids that give less than 100% reaction by the procedure of Yemm and Cocking (15) were found to give 100% reaction in the system described here (Tyrosine, for example), or to more nearly approach 100% color formation, such as asparagine.

The exceptions to 100% color formation are minor, and do not result in gross inaccuracies in analysis of beet juices. Gamma-amino-butyric acid gives about 90% as much color as glutamic acid. In the total amino acid spectrum of the sugar beet, the error introduced is insignificant.

Asparagine gives about 63% as much color as glutamic acid, on an equivalent amino nitrogen basis. Carruthers et al. (2) found asparagine generally less than 1/10 as abundant as glutamine in raw juice. On many California beets we have found slightly higher proportions. Nonetheless, even in raw juice, the error introduced could scarcely exceed 4%. In processing, asparagine is nearly all converted to aspartic acid, which gives 100% color formation in this analysis. Goodban et al. (4) report only traces

of asparagine in diffusion juice and molasses. Freed and Hibbert (3) show about .07% asparagine or dry matter in thin and thick juices. It is believed, therefore, that small amounts of asparagine existing in beet juice will contribute but minute errors in the determinations for total amino nitrogen. In determination of total amino plus PCA nitrogen, asparagine would convert to asparatic acid to give 100% color formation.

The procedures set down here are not inflexible. Adaptations can be made to suit laboratory conditions or preferences. For example, the curve can reach far beyond the 10 gamma limit by greater dilution of the reactants so long as standards have been set up exactly the same way. Volumes used here may be varied. It seems only important that enough ninhydrin be present; that there be not too much KCN; that pH is proper and that samples are run exactly as are standards. Carruthers *et al.* (1) have preferred to carry out the alkaline hydrolysis for the PCA determination on a more concentrated sample in a large volume, which is then diluted further and the Moore and Stein procedure applied to an aliquot of the diluted hydrolysate.

In this laboratory, extensive use of the method led to a time saving adaptation that eliminates the necessity of diluting samples containing less than 1.0 mg amino N per ml. Discs of about 8 mm diameter are bored from a pad of filter paper. Pressure of the drill press makes the discs convex. These are laid, convex side up, on a clean surface and a 10 lambda aliquot of the sample is applied with a micro-pipette. The disc will adhere to the tip of the pipette as the sample begins to absorb, which allows it to be lifted over the mouth of the test tube before any of the sample reaches the periphery of the disc. When all the sample has been drawn out, the impregnated disc falls into the test tube. This may be left indefinitely until analysis is ready to be run. If only amino N is being run, 1 ml of water is added and the analysis completed normally. If amino plus PCA nitrogens are being run, hydrolysis is done with $\frac{1}{2}$ ml of 20% NaOH or with a little water and the usual $\frac{1}{4}$ ml of 40% NaOH.

Thus far there has been no occasion in this laboratory to apply the impregnated disc technique in the determination of Total Nitrogen. One would expect the technique to be usable. However, higher blank readings might occur, and certainly a longer digestion time would be required.

The digestion for Total Nitrogen may be carried out in large pyrex test tubes (1"×8") and the digest washed into 100 ml Kohlrausch flasks for neutralization. In this manner a clamp arrangement for the tube allows up to 18 simultaneous digestions on a 6 unit electric digestion apparatus.

Volume measurement of the digestion salts is recommended for speed. Suitable measuring devices are easily made.

Several metallic cations interfere with the ninhydrin reaction in an n-butanol system, including the mercuric ion (7). However the digestion mixture used here does not effect the color formation in the cellosolve system. Copper very markedly inhibits the reaction and so cannot be used as a digestion catalyst.

As in the amino N determination, the KCN and ninhydrin may be added separately (1ml of .0002M KCN and 0.5 ml of 10% ninhydrin) to give the same color as the mixture.

Here again some deviation from the procedure as described are possible. For example, it is not essential to use 10% ninhydrin. However, with the stronger solution, color formation has nearly ceased after 15 minutes; so there is less chance of error in the time element.

Summary

A colorimetric method for determining total amino nitrogen is described wherein a simple 2-component acetate buffer is used. The reaction is carried out in a buffered methyl cellosolve system with ninhydrin and KCN. Most amino acids, on an equivalent nitrogen basis, show the same amount of color formation after 15 minutes in a boiling water bath when read at 570 millimicrons.

A method is described for including pyrrolidone carboxylic acid in the total amino determination by using the NaOH component of the buffer in a brief hydrolysis step, followed by the addition of the acetic acid component and carrying out the remainder of the amino-N procedure.

A method is described for the determination of total nitrogen by using a micro-digestion procedure, neutralizing and diluting the digestion mixture, and then performing the ninhydrin reaction on an aliquot of the diluted digest.

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