Analysis of Protein and Total Usable Nitrogen in Beer and Wine Using a Microwell Ninhydrin Assay

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ABSTRACT


In this study we present a ninhydrin based microwell assay that can be utilized in place of the traditional Kjeldahl method for the determination of the protein content of beer or wine. In addition, the assay is ideal for the determination of free amino acids in beer (FAN), a term understood and used by brewers, and yeast assimilable nitrogen (YAN) used by enologists. The assay only measures alpha amino acids and ammonia so other nitrogen sources are not detected, resulting in a 30% reduction in total protein of a variety of beers compared to the Kjeldahl method, which measures nitrogen from all sources. The results also showed that only 25% of the total “protein” in beer is actually derived from peptides larger than 3,500 Kd. Analysis of beer or wine with the microwell assay for total usable nitrogen was compared to the standard FAN and YAN methods and conditions were determined for maximal efficiency and precision. Superior results were obtained with low reaction volumes and a stable sodium acetate buffered ninhydrin reagent at pH 5.5. As an alternative, for use with cuvettes, a reduced volume FAN assay using the same pH 5.5 sodium acetate buffered ninhydrin reagent gave comparable results. The assay is economical, rapid, accurate and applicable to large numbers of samples.

Key words: beer, FAN, protein, wine, YAN

INTRODUCTION

Proteins contained in beer are for the most part derived from water soluble proteins contained in the grains used in the malting and brewing process. These proteins are important determinants of beer quality, yet most proteins are modified or lost during the malting process. Various proteins and polypeptide fragments from malt extracts have been identified by mass spectroscopy, but in barley proteins and polypeptide fragments from malt extracts are modified or lost during the malting process. Various important determinants of beer quality, yet most proteins in the malting and brewing process. These proteins are from water soluble proteins contained in the grains used in barley endosperm and the 40 kDa protein Z. Both of protein (LTP1) which is found in the aleurone layers of these are mainly represented by the 9 kDa lipid transfer proteins in beer that are introduced from malt or cereal adjuncts such as proteases, hordeins, serpeins and others, are to a large extent processed to peptides and free amino acids during mashing. Added to the total nitrogen content of beer are nucleic acids and other non-protein derived nitrogen sources. The values obtained for the protein content of beers are almost exclusively based on Kjeldahl assays which are complicated, require specialized equipment, use relatively large quantities of beer and measure total nitrogen and not specifically nitrogen derived from protein, peptides or amino acids.

The fermentation of beer and wine is conducted with various strains of yeast, usually Saccharomyces, which convert sugars to ethanol and carbon dioxide. The survival and growth of the yeast requires the removal of the amino group from any alpha amino acid or ammonia, which is then used to form other amino acids required for protein synthesis. The source of the amino acids in beer is derived from the extraction of free amino acids from the mash or the enzymatic hydrolysis of proteins and peptides during the formation of the wort. The nitrogenous constituents of wort include amino acids, peptides, proteins, nucleic acids and their degradation products. With wine, grape juice/must provides the main source of nitrogen necessary for yeast growth and fermentation and contains the same complex mixture of nitrogenous compounds as wort. Of particular importance is the evaluation of available nitrogen status of the juice/must prior to conducting the fermentation. Sub-optimal concentrations of available nitrogen are associated with lagging and incomplete fermentation, and sulfide evolution. In the stationary phase of yeast development only low levels are required as a fermentation stimulant of the yeast while higher levels are required during the growth phase.

There is some confusion in the literature with the terminology used to express the useable nitrogen available for yeast during fermentation. Total nitrogen values are obtained from the sum of all nitrogenous compounds present and are usually determined by the Kjeldahl method. Free Amino Nitrogen (FAN), by definition, reflects the amino acid nitrogen available to yeast during fermentation. In reality, FAN is a measure of total usable nitrogen since the ninhydrin based assay measures ammonia as well as small peptides as outlined by the 1977 European Brewery Convention Methods of Analysis. Yeast Assimilable Nitrogen (YAN) was initially defined as only alpha amino acid derived nitrogen. However, it is now considered a measure of both free alpha amino acids and ammonia, and is the sum of two assays. Ammonia can be meas-
ured using the 2-oxoglutarate method, while amino nitrogen may be measured using the formol titration method of Shively and Henick-Kling, or by the NOPA method of Dukes and Butzke. Total Usable Nitrogen or Usable Nitrogen is defined as nitrogen compounds that can be assimilated or metabolized by yeast during fermentation and includes free alpha amino acids, ammonia, and small peptides.

In the following study, we modified a ninhydrin microwell method used to measure total protein in tissue hydrolysates for the quantitation of the protein and total usable nitrogen content of beer or wine. The method was compared with standard methods used in the beer and wine industries.

**MATERIALS AND METHODS**

Beer samples representing Domestic and Foreign Pilsner, Foreign Premium Pilsner, Domestic Light Pilsner, dry Stout, Bock and Craft beers representing several styles were obtained for protein or FAN analysis. They were opened and kept in sterile containers at 4°C overnight prior to analysis. For the analysis of dialyzed beer, 200 μL of beer was placed in microdialyzer vessels (Pierce, Rockford, Ill) with a molecular weight cut-off of 3,500. The vessels were floated in a foam holder on distilled water with several changes of water over a 4 h period, allowing only the surface of the membrane to touch the water in order to maintain minimal dilution of the beer. For larger volumes required for Kjeldahl analysis, the beer was dialyzed in dialysis membranes with a 3,500 molecular weight cut-off.

For hydrolysis, each sample of beer or dialyzed beer (200 μL) was added to 1.5 mL microfuge tubes and mixed with 200 μL of 12 N HCl. The tubes were closed and heated for 18 h at 95°C, cooled and microfuged. Samples analyzed in the assay were removed from the acid hydrolysate without evaporation of the 6 N HCl. If significant evaporation occurred, water was added to bring the volume back to 400 μL.

**Reagents for the microwell assay**

**Ninhydrin stock solution:** Ninhydrin (8 g) was dissolved in 300 mL of ethylene glycol and 100 mL of 4 N sodium acetate pH 5.5 buffer (544 g sodium acetate tetrahydrate and 400 mL glacial acetic acid in 1 L water). This solution was stable at room temperature for at least 6 months.

**Stannous chloride solution:** SnCl₂ (500 mg) was dissolved in 5 mL ethylene glycol. This solution was stable at room temperature for at least 6 months.

**Ninhydrin reagent/working solution:** Prior to the assay, 25 μL SnCl₂ solution was added for every 1 mL of ninhydrin stock solution and mixed well.

**Protein standard (1 μg/μL):** Twenty milligrams of bovine serum albumin (Sigma Chemical Co, St Louis MO) was hydrolyzed in 500 μL of 6 N HCl in a sealed microfuge tube at 100°C for 18 h. The hydrolysate was microfuged and the supernatant brought to exactly 20 mL with distilled water.

**Glycine nitrogen standard:** Glycine (107.2 mg) was dissolved in distilled water and brought to exactly 100 mL.

For protein analysis of un-dialyzed beer, 3 μL of each beer hydrolysate was added in duplicate to Fisher 96 well microwell plates and 100 μL of working solution added to each sample. Dialyzed beer required 8 μL of hydrolysate for the assay. The blank consisted of 100 μL working solution. A standard consisting of 5 μL of the protein standard was carried in duplicate with each assay. The plate was incubated at 104°C for 10 min using a dry titer plate heater (Fischer Scientific Fairlawn, NJ, USA). Net optical density (OD) readings were converted to micrograms protein based on the OD readings from the 5 μg standard. For the calculations it must be kept in mind that the original beer sample was diluted in half with the addition of an equal volume of 12 N HCl. For more dilute protein samples it was possible to use up to 10 μL of hydrolysate without the need to evaporate the HCl and re-suspend the sample in a smaller volume of water.

The standard FAN assay was performed as previously described in the 1977 EBC methods of analysis or modified for reduced volume as described below.

**Ninhydrin color reagent:** Four grams of anhydrous Na₂HPO₄, 6 g KH₂PO₄, 0.5 g ninhydrin and 0.3 g fructose were dissolved in a total of 100 mL distilled water.

**Glycine standard:** Glycine (107.2 mg) was dissolved in distilled water and brought to exactly 100 mL. This solution was stable at 4°C for two weeks.

**Dilution reagent:** Two grams of potassium iodate (KIO₃) was dissolved in 600 mL distilled water and 400 mL 96% ethanol was added.

For the standard FAN assay, 1 mL of beer was diluted to 50 mL with distilled water and 2 mL of the diluted beer was transferred to a 16 x 150 mm test tubes. Ninhydrin color reagent (1 mL) was added and the tubes were heated in a boiling water bath for 16 min. The tubes were transferred to a cold water bath and 5 mL of dilution reagent added, mixed and absorbance recorded at 575 nm against a blank containing 2 mL of water in place of the sample.

The reduced volume FAN assay (FrAN pH 6.8) was conducted using the phosphate buffered ninhydrin reagent or the sodium acetate buffered ninhydrin reagent (FrAN pH 5.5) as follows:

**FrAN pH 6.8:** Beer (30 μL) or 20 μL glycine standard (4 μg N) was added to 2 mL distilled water and 1 mL of pH 6.6 phosphate buffered ninhydrin color reagent in glass test tubes, vortexed and placed in a boiling water bath for 16 min. The tubes were then cooled in a cold water bath and the absorbance at 575 nm recorded against a blank containing 30 μL of water in place of the sample.

**FrAN pH 5.5:** Beer or grape juice (30 μL) or 20 μL glycine standard (4 μg N) was added to 200 μL of the pH 5.5 acetate buffered ninhydrin reagent and placed in a boiling water bath for 10 min. After 10 min the samples were removed and 2.8 mL cold water added, the tubes vortexed and the absorbance at 575 nm recorded against a blank containing 30 μL of water in place of the sample.

For the microwell FAN assay, 2 μL glycine standard (0.4 μg N) or 2 μL of beer or grape juice was added to a 96 well microwell plate and 100 μL of the pH 5.5 acetate buffered ninhydrin reagent added and the plate heated for
10 min at 104°C. The absorbance was then recorded at 575 nm on a microwell plate reader.

To conduct the YAN assay in grape juice or wine, the ammonia (rapid) kit and primary amino nitrogen kits were obtained from Megazyme (Wicklow, Ireland) and the assays performed according to the manufacturer’s instructions.

RESULTS

In order to analyze the hydrolyzed protein samples without the need for volume adjustments, it was necessary to prevent significant loss of sample volume during hydrolysis. The hydrolysis was performed with a number of microfuge tubes from a variety of manufacturers and varying conditions. Maximum recovery of amino acids was obtained within 18 h at 98°C. Temperatures exceeding 105°C resulted in excessive charring and variable loss of volume. Microfuge tubes (1.5 mL) that were found acceptable were Fisher locking lid types, Fisher low retention tubes and Eppendorf safe-lock tubes. It was also possible using the same protocol to scale the method down to 0.5 mL PCR tubes using 50 µL beer and 50 µL 12 N HCl.

Some beer proteins contain significant amounts of proline or hydroxyproline which could reduce the color yield since imino acids do not give the typical absorbance of alpha amino acids. Absorbance at 575 nm of hydrolysates of several proteins at varying concentrations including gliadin and collagen are shown in Fig. 1. There was some variation between proteins but no indication that proteins high in imino acids gave reduced color yields per unit weight. The average absorbance with standard deviation for 5 µg of the 6 different proteins at 575 nm was 1.62 ± 0.24.

The protein content of seven different styles of beer was analyzed to show the potential and reproducibility of this assay for the analysis of beer protein (Fig. 2). Inter assay variability for triplicate samples was very low for each beer analyzed, with the protein content for different beers ranging from 1.60 ± 0.03 mg/mL for the Domestic Light Pilsner to 2.61 ± 0.07 mg/mL for the Foreign Premium Pilsner. The Domestic Pilsner Light and the Domestic Pilsner were both significantly lower in protein content than the other five beers (<P 0.005).

It was important to know whether the values from the microwell method agree with the Kjeldahl method, which is the industry standard, and what percentage of the amino acid derived nitrogen in beer is actually due to protein and not free amino acids and small peptides? To answer these questions we dialyzed each beer against 3,500 MW cut off membranes, hydrolyzed the retained protein and assayed with both the Kjeldahl and ninhydrin methods. These results, shown in Fig. 3, indicate that the Kjeldahl method of analysis gave on average 30% higher protein values than the ninhydrin assay for undialyzed beer. When the beers were dialyzed prior to hydrolysis, both analytical procedures showed a significant reduction in protein values, averaging close to 75% of the “protein” nitrogen removed during dialysis.

![Fig. 1. Absorbance values for proteins with high imino acid content compared to standard proteins. Albumen (□), soybean trypsin inhibitor (■), collagen (●), gliadin (○), lima bean trypsin inhibitor (▲), elastin (△). Collagen and gliadin, proteins high in proline or hydroxyproline, showed no reduction in absorbance at 575 with the microwell ninhydrin analysis.](image)

![Fig. 2. Analysis of beer protein using the titer plate ninhydrin method. Values shown are the average of 3 analyses on each beer with standard deviation. The beers analyzed were: Domestic Pilsner (A); Domestic Light (B); Foreign Pilsner (C); Foreign Stout (D); Foreign Premium Pilsner (E); Domestic Malt Liquor (F); and Bock (G).](image)
A wide variety of beers were compared for free amino acid nitrogen using the standard FAN and reduced volume FAN assay and the microwell method (Table I). Although the values were comparable, there was a discrepancy for some beers with the standard FAN method yielding lower values in general. We explored these assays further to see if there was an explanation for this discrepancy.

The standard FAN assay has a total volume of 8 mL, while the reduced volume assay 3 mL and the microwell assay only 100 μL. This makes direct comparisons difficult when comparing beer samples in terms of color yield per unit nitrogen. To compensate for the difference in volumes we used 2.67 times more sample for the standard FAN assay than the reduced volume FAN assays. The microwell assay was compared independently, using sample nitrogen comparable in color yield to the other assays.

The linearity of glycine and beer with increasing nitrogen concentration in the four assays were compared after 16 min incubation (Fig. 4). A U.S. domestic pale ale, which was used along with the standard glycine nitrogen in each method, is shown as a dotted line. All reactions were linear with increasing nitrogen concentration, however the standard FAN assay and the rFAN pH 6.8 gave significantly lower absorbance values than assays using the pH 5.5 ninhydrin reagent. This is illustrated in Table II which shows the average absorbance per microgram glycine nitrogen for these assays over the range of concentration.

Figure 5 shows the reaction rate of the different ninhydrin based assays using 6 μg for the rFAN assay, 16 μg for the standard FAN assay and 0.4 μg for the microwell assay. The standard FAN and the rFAN pH 6.8 reactions required at least 16 min to reach maximum absorbance values while the rFAN pH 5.5 and the microwell assays were more rapid, requiring less than 8 min to reach maximal values. The same rates of reaction were observed for beer samples as were observed for the glycine standard.

![Graph showing comparison of total protein in beer analyzed by the ninhydrin and Kjeldahl method of analysis.](image1)

**Fig. 3.** A comparison of total protein in beer analyzed by the ninhydrin and Kjeldahl method of analysis. Un-dialyzed beer shown in black bars and dialyzed beer in white bars. The ninhydrin (N) and Kjeldahl (K) methods of analysis are shown for each beer. Domestic Pilsner (A); Domestic Light (B); Foreign Pilsner (C); Foreign Stout (D); Foreign Premium Pilsner (E); Domestic Malt (F); and Bock (G).

**Fig. 4.** Linearity of the FAN and microwell assays with increasing amounts of glycine standard and beer. The solid line represents glycine for the standard FAN assay (○), the reduced FAN assay pH 6.8 (□), the reduced FAN assay pH 5.5 (■) and the microwell assay (△). Dotted lines represent increasing amounts of beer for the same assays to roughly match the increasing microgram amount of standard nitrogen and to confirm the linear assay response of beer (complex matrix) in comparison to glycine. Since the microwell assay is on a different scale of sensitivity (6–7 times more sensitive), the actual OD values for this microwell assay have been multiplied by a factor 6.5 in order to compare on the same graph with the other assays.

**Table I.** Free amino nitrogen (mgN/L) comparison between FAN and reduced volume FAN assay and the microwell method.

<table>
<thead>
<tr>
<th>Beer</th>
<th>Microwell</th>
<th>FAN</th>
<th>Reduced Vol FAN pH 6.8</th>
<th>Reduced Vol FAN pH 5.2</th>
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<tr>
<td>A</td>
<td>169</td>
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<td>O</td>
<td>70</td>
<td>52</td>
<td>96</td>
<td>83</td>
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</table>

**Table II.** Sensitivity of the microwell FAN assay compared to cuvette-based FAN assays.

<table>
<thead>
<tr>
<th></th>
<th>Absorbance 575 / μg nitrogen*</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAN</td>
<td>0.100 ± 0.005</td>
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<tr>
<td>rFAN pH 6.8</td>
<td>0.303 ± 0.049</td>
</tr>
<tr>
<td>rFAN pH 5.5</td>
<td>0.452 ± 0.017</td>
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<tr>
<td>microwell FAN</td>
<td>2.945 ± 0.072</td>
</tr>
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*Values are the mean of 3 determinations with standard deviation.

rFAN is the reduced volume FAN assay as described in methods.
DISCUSSION

Almost all packaged foods indicate the protein content on the label or the information is available to the consumer and for beer this is no exception. The industry standard has always been the Kjeldahl nitrogen analysis. In the present study we have presented an alternative method with several advantages over the Kjeldahl method.

The microwell ninhydrin assay gives lower protein values than the Kjeldahl method due in large part to the ninhydrin reaction being specific for amino acids and ammonia, while the Kjeldahl measures all nitrogen containing components. The interpretation of protein content is compounded by the evidence that over 75% of the “protein” in beer is not actually protein, but free amino acids and peptides, either extracted from the plants, or derived from proteins during the brewing process. In the studies presented here we considered that all amine-containing molecules less that 3,500 Kd in size were not intact proteins.

Another possibility for the lower protein value in beer with the ninhydrin assay is that beer contains hordein proteins with a high content of proline. This might result in a lower than predicted ninhydrin value, since imino acids have an absorbance maximum at 440 nm and give a much reduced color value at 575 nm compared to alpha amino acids. This was apparently not the case since collagen, which contains 30% proline plus hydroxyproline, and gliadin which contains 10% proline, when assayed with the ninhydrin method showed no diminution of color yield per milligram of protein compared to other proteins.

The accepted values for the protein content of beer are actually somewhat lower than the true protein content, as most of the protein has been converted to peptides and amino acids. If however, we consider the nutritive value of amino acids, peptides and proteins in beer, it is immaterial what form the amino acids are in. The fact that ninhydrin reaction does not measure imino acids is of no consequence, as they have no nutritive value. The ninhydrin assay that we present here gives an accurate and simple means of measuring the total ammonia and alpha amino derived nitrogen in beer.

The conditions for the reaction of alpha amino acids with ninhydrin were explored extensively in the initial studies of Moore and Stein12. The reduced hydridantin of ninhydrin is required for complex formation. In the oxidative deamination of amino acids by ninhydrin, 1 equivalent of the reagent is reduced during the formation of diketohydrindylidene-diketohydramine15. In a complete absence of oxygen there would be no requirement for additional hydridantin. However, this is not the case and unlike alpha amino acids, NH3 does not give rise to the reduced ninhydrin, which is essential for the formation of the colored complex. The addition of a reducing agent such as fructose or stannous chloride provides the additional hydridantin that is required. The hydridantin is only slightly soluble in aqueous solvents, so in order to maintain optimal levels of reagents, an organic solvent such as ethylene glycol is required. The pH is important and for maximal and comparable ninhydrin color yields between amino acids a pH of 5.5 is optimal. Ammonia, for example, gives less than 75% absorbance at pH 6.8 as it does at pH 5.5. A reaction temperature of at least 95°C is required to reach maximum color yield within a reasonable time. The rate of the reaction is dependent on the concentration of reagents as well as the temperature.

The standard FAN assay uses a phosphate buffered ninhydrin reagent at pH 6.6 to 6.8 which is unstable after two weeks storage. The pH is not ideal and results in a lower absorbance spectral peak and a lower color yield. As a result of diluting the reagents, the reaction requires longer incubation times for completion. All of these can be overcome and still make use of a cuvette for reading the reaction by simply reducing the total reaction volume and using the sodium acetate buffered ninhydrin reagent at pH 5.5. This reaction is very rapid, reaching maximum absorbance within 5 min. A marked negative effect of reagent dilution was observed if we diluted the rFAN pH 5.5 assay to 3 ml prior to heating. Under these conditions
the change in OD/µg N was lowered to 0.038 ± .002. On the other hand, if we conducted the rFAN pH 6.8 assay in 3 mL rather than diluting to 3 mL after boiling we had much higher net absorbance readings. This was due in part to a marked shift in the reaction color from purple to red with very high blank readings.

In the wine industry it is important to know the available nitrogen for the controlled growth of yeast. This is usually determined by a YAN method that requires two independent assays, free amino acids and ammonia. The microwell ninhydrin or the reduced volume FAN pH 5.5 assay measured both of these in a single determination with similar results. Since proline is not utilized by yeast, it is not a relevant factor in these analyses.

In summary we present a microwell ninhydrin assay that is suitable for determining protein as well as total usable nitrogen in beer or grape juice/wine. The assay is inexpensive, rapid, accurate and applicable to large numbers of samples. There is a considerable saving in time as all sample absorbance measurements are made with a single reading. As an alternative, for use with cuvettes, the reduced volume FAN assay using the pH 5.5 sodium acetate buffered ninhydrin reagent gives comparable results. The new reduced volume assay should be useful to most brewing and enology labs for the routine determination of FAN (or total usable nitrogen) and in providing truer values of protein in beer and wine.

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