A new way to test the free amino nitrogen content in alcoholic beverages with the SPECTROstar® *Nano*

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- **Ninhydrin-based assay detects free amino nitrogen (FAN) in beverage and food analysis**
- **Scaling down the original assay reduces reagents and limits waste**
- **SPECTROstar® *Nano* is the optimal reader to measure absorbance in cuvettes and microplates**

Introduction

Standard Brewing quality control analyses include the test for free amino nitrogen (FAN). This allows an estimation of the protein-content and is a beer quality indicator. The traditional way to determine the protein content of beers is based on Kjeldahl assays which are elaborate and quite expensive. An alternative to this assay is the ninhydrin-based FAN assay for cuvettes. A standard assay used for 40-50 years by the brewing industry. Primary amino acids can be detected with the help of ninhydrin. The result is a blue dye that shows an absorbance maximum at about 570 nm (Fig. 1). The amino acid glycine is used as a standard substance for this assay.

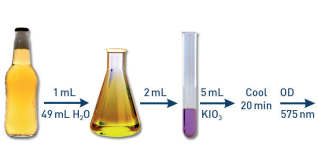
The next step was to reduce the volume for the standard cuvette assay and to move to a multi-well format. This was pioneered by Starcher and staff and our lab, and it was published in the brewing literature. Scaling down the test reduces the demand for expensive reagents, it limits waste and thus makes for simpler and less expensive disposal.

The SPECTROstar *Nano* from BMG LABTECH combines cuvette and microplate measurements in one instrument. The integrated spectrometer can take spectra over time and the MARS data evaluation software shows overlay plots of all measurements.

Materials & Methods

- Clear 96-well plates from Greiner
- SPECTROstar *Nano*, BMG LABTECH

Standard FAN assay at pH 6.8:



Ninhydrin Color Reagent: 4 g anhydrous Na_2HPO_4, 6 g KH_2PO_4, 0.5 g ninhydrin and 0.3 g fructose are dissolved in a total of 100 mL distilled water. Stock may be stored refrigerated up to 2 weeks in an amber bottle.

Glycine Standard Stock: Exactly 107.2 mg glycine is dissolved in distilled water and brought to exactly 100 mL. Standard stock may be stored at 0 - 4°C to avoid growth of molds.

Glycine Standard Solution: 1 mL glycine standard stock is diluted into 100 mL (final vol.) with distilled water. The standard contains 2 mg amino-nitrogen/L. Use freshly made for each daily assay run.

Dilution solution: 2 g of potassium iodate (KIO_3) is dissolved in 600 mL distilled water and 400 mL 96% ethanol added. Stable long-term at 4°C; use close to room temperature to prevent condensation on cuvettes.

Assay: 1 mL of beer is diluted to 50 mL with distilled water and 2 mL transferred to 16 X 150 mm test tubes. Ninhydrin color reagent (1 mL) is added and the loosely covered tubes heated in a boiling water bath for 16 min. The tubes are transferred to a cold water bath to bring to 20°C (within 20 minutes) and then 5 mL of dilution reagent is added, mixed and the absorbance immediately recorded at 575 nm against a blank containing 2mL of water in place of the sample.

Microplate FAN assay at pH 5.5

Ninhydrin Stock Solution: 8 g of ninhydrin is dissolved in 300 mL of ethylene glycol and 100 mL of 4 N sodium acetate pH 5.5 buffer* is then added (*544 gm. sodium acetate tetrahydrate + 40 mL glacial acetic acid in 1L water). Stock is stable at room temperature for at least 6 months.

Stannous Chloride Solution: 500 mg $SnCl_2$ is dissolved in 5 mL ethylene glycol. Solution is stable at room temperature for at least 6 months.

Ninhydrin Reagent/Working Solution**: Prior to the assay, 25 µL of $SnCl_2$ solution is added to every 1 mL of ninhydrin stock solution and mixed well.

Assay: 2 µL (0.4 µg N) of glycine standard or 2 µL of beer or grape juice is added to a 96 well micro-well plate and 100 µL of the pH 5.5 acetate buffered ninhydrin reagent is then added. The plate is heated for 10 min at 104°C. The absorbance is recorded simultaneously for multiple samples at 575 nm in the SPECTROStar *Nano* microplate reader.

Reduced volume cuvette FAN assay at pH 5.5

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

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Controls and calculations

Tests should be in duplicate or triplicate for each sample, water blanks, and glycine standard. The average blank value is subtracted from each sample and the standard glycine. Then the blank corrected sample net (average) absorbance is divided by the net absorbance of the standard glycine and multiplied by 2 and the dilution factor (50 for beer diluted 1 mL into 50 mL or 100 for beer wort diluted 1 mL into 100 mL) to obtain FAN content in ppm. (Samples, including mash samples, should be free of haze/ debris before dilution – filter or centrifuge.)

Results & Discussion

Wavelength Optimization

The optimal wavelength to measure this assay can be obtained by the scanning capabilities of the spectrometer in the SPECTROstar *Nano*. Spectra were taken from the ninhydrin reaction either with glycine or a beer sample (Fig. 1).

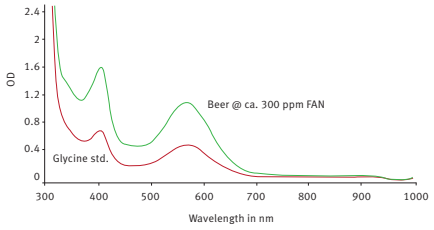

Fig. 1: Spectra captured for a glycine standard (red line) and a beer sample (green line).

The spectra taken with the SPECTROstar *Nano* proved that there is a clear absorbance maximum at about 570-575 nm.

Reaction times required for completion of the FAN assays

The reaction times of 4 different ninhydrin-based FAN assays were compared. Next to the already explained three assays (standard FAN pH 6.8, microplate FAN pH 5.5 and reduced volume rFAN cuvette pH 5.5, please see Materials and Methods) a second reduced cuvette FAN assay at pH of 6.8 was investigated. The result is shown in Fig. 2.

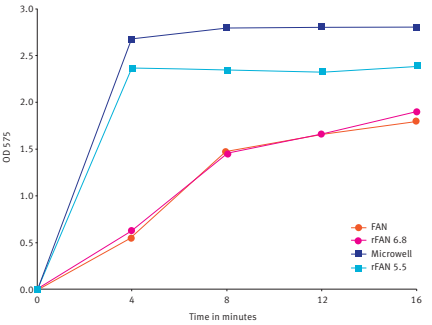

Fig. 2: Reaction times required for completion of 4 different FAN assays).

It turned out that the reaction rates are different for the assays. It is seen that the standard FAN and rFAN pH 6.8 reactions required at least 16 minutes to approach, at best, maximum absorbance. The rFAN pH 5.5 and the microplate FAN assay attaining maximal values and stable plateaus within 8 minutes. There was no loss of absorbance in any of the assays when incubated up to 20 minutes.

Conclusion

We showed that the SPECTROstar *Nano* was very well suited to measure the FAN microplate and cuvette assays for beer samples. The new reduced volume cuvette and microplate based FAN deal with a more concentrated ninhydrin stock solution, no extended cooling time is needed and no dilution/quench solution is required. There is further no need to run dilutions on beer/wort samples prior to sampling. These are all advantages compared to the 40-50 years old standard FAN cuvette assay.

The assays were optimized further in terms of pH and buffers and should prove useful for many brewing, enology, distilling and food laboratories for the routine determination of free or total useable nitrogen.