

## RAPID SPECTROPHOTOMETRIC MEASUREMENT OF SOLUBLE NITROGEN IN MICROMALTS FROM A BARLEY BREEDING PROGRAMME

BY R. M. HASLEMORE

(New Zealand Institute for Crop and Food Research Limited, Private Bag 11030, Palmerston North, New Zealand)

AND A. A. GILL

(Analytical and Applied Research Group, Plant Breeding International—Cambridge Ltd, Trumpington, Cambridge CB2 2LQ, United Kingdom)

Received 2 May 1995

**A spectrophotometric method for determining soluble protein has been applied to the measurement of soluble nitrogen (SN) in micromalts from a barley breeding programme. A regression equation between wort SN values, measured by the Kjeldahl method, and the absorbance difference ( $A_{215}-A_{225}$ ) of diluted wort samples was prepared and then used to predict SN levels of 353 worts produced from breeding material from a diverse genetic background. These data were highly correlated with those obtained from the Kjeldahl method ( $r = 0.952^{***}$ ).**

**A second calibration equation relating wort absorbance at a single wavelength ( $A_{215}$ ) to Kjeldahl nitrogen data was able to predict wort SN values with equal accuracy compared with the relationship based on absorbance difference ( $A_{215}-A_{225}$ ). Here, the correlation between the two data sets was  $r = 0.953^{***}$  ( $n = 353$ ). The spectrophotometric method is a rapid and simple means of measuring wort SN in the large numbers of samples generated by barley breeders and the single wave-length measurement offers a particularly efficient method of screening the very small wort volumes produced from test tube-scale mashes.**

**Key Words:** *Barley breeding, wort nitrogen, Kjeldahl, UV absorption.*

### INTRODUCTION

Malting barley contains about 9–12% protein, usually expressed as % nitrogen, most of which remains in the grain following malting. About 30–40% of this protein is dissolved into the wort during the mashing phase of brewing, the precise proportion being of interest to the brewer as it influences yeast nutrition, foam properties, alcohol yield and haze potential. This fraction of the total malt protein, generally called soluble nitrogen (SN), is affected by the malting and brewing conditions used and is also influenced by barley variety. A convenient and precise means of assessing the SN concentration of micromalts prepared from breeding lines would therefore help the barley breeder. The SN value can be used with the total barley nitrogen concentration, rapidly measured by near infrared reflectance, for example, to give an indication of soluble nitrogen ratio (SNR) or Modification Index.

There are several direct methods for the determination of nitrogen in biological materials, including variations of the Kjeldahl procedure<sup>2,6</sup>, and the Dumas combustion method<sup>2</sup> now being used more widely. Both have limitations in that they are time consuming, require expensive equipment, and in the case of the Kjeldahl method, use corrosive reagents and toxic catalysts. There is an alternative, simple and sensitive spectrophotometric method<sup>10</sup> for measuring dissolved protein based on absorption in the UV region of the spectrum. Protein adsorption is often measured at 280 nm but the response depends upon the aromatic amino acid composition of the proteins present and is subject to interference from nucleic acids and nucleotides.

Waddell<sup>10</sup> showed that a strong absorption peak between 250 nm and 200 nm was caused by peptide bonds and so was independent of amino acid composition. He further demonstrated a linear relationship between protein concentration and the difference in absorbance between 215 nm and 225 nm

( $A_{215}-A_{225}$ ) and noted that this difference largely negated interferences when using plasma samples. Franken-Luykx<sup>4</sup> reported a linear relationship between absorbance difference ( $A_{215}-A_{225}$ ) and SN in unhopped and end-fermented worts from malts of different barley varieties. More recently, both the European Brewery Convention<sup>7</sup> (EBC) and the American Society of Brewing Chemists<sup>1</sup> (ASBC) have assessed the method with unhopped worts in collaborative trials and both organisations have recommended its use, although the EBC have added the proviso that the spectrophotometric method should not be used as a basis for commercial transactions.

Barley breeders are now paying more attention to protein degradation during malting and mashing in response to current demands for barley varieties with a much wider range of protein modification characteristics during processing. However, the spectrophotometric method of measuring SN is apparently not being used as a routine method for assessing breeding material despite its simplicity and convenience.

This paper describes the preparation of a calibration equation relating wort SN levels by the Kjeldahl method to absorbance difference values ( $A_{215}-A_{225}$ ) and its subsequent performance in determining SN in worts from micromalts prepared from spring and winter barley breeding lines. The possibility of using a single wavelength measurement was evaluated as an even more rapid method for routine use in screening breeding lines.

### EXPERIMENTAL METHODS

#### *Material*

Grain samples were taken from winter and spring breeding trials, ranging from the F<sub>3</sub> generation to commercial varieties, grown in the 1993–94 season on five sites near Cambridge. 20 g samples of cleaned and sieved grain were micromalted using a Phoenix Automatic Micromalting system and 10 g malt samples were mashed by a modified Institute of Brewing procedure described by Gothard *et al.*<sup>5</sup>

### Measurement of Soluble Nitrogen (SN)

5.00 ml of each wort sample was digested and SN was determined according to the method of Starr and Smith<sup>9</sup>. A further aliquot of wort (1.00 ml) was diluted to 100 ml with sodium chloride solution (5.0 g/litre) using volumetric flasks. Absorbance at 225 nm, then at 215 nm, was measured in quartz cuvettes using a Perkin Elmer Lambda 3B UV/visible spectrophotometer. At each wavelength, the spectrophotometer was zeroed using dilute sodium chloride.

For further information on sound operating practice, see the EBC Notes on Procedure referred to by Lie<sup>7</sup>.

### Calibration set

A set of malt samples was chosen to represent as wide a range as possible of wort SN levels and then mashed in duplicate. SN levels in the duplicate worts were then measured spectrophotometrically and by the Kjeldahl method. A regression equation describing the relationship between mean values of absorbance difference ( $A_{215}-A_{225}$ ) and SN was calculated.

### RESULTS AND DISCUSSION

Figure 1 shows absorbance levels of a typical wort sample in dilute NaCl solution scanned between 190 nm and 305 nm in which the peak between 230 nm and 190 nm is attributed to peptide bonds. The peak gave maximum absorption at 201 nm and the slope from 225 nm to 215 nm was essentially linear and was reported to be directly proportional to nitrogen concentration<sup>10</sup>.

To test this for wort samples, a calibration set was prepared from 31 micromalt samples with a range of wort SN levels from 352 to 642 mg/litre; and an absorbance difference ( $A_{215}-A_{225}$ ) range of 0.139 to 0.279. Least squares regression analysis gave a relationship of:

$$\text{Wort SN (mg/litre)} = 2249 (A_{215}-A_{225}) + 30$$

between these data sets with a correlation coefficient ( $r$ ) of 0.988\*\*\* ( $n = 31$ ). These data are illustrated in Figure 2.

This calibration equation was then used to predict SN in wort samples generated from other micromalts produced from a range of breeders' lines grown on different sites, and covering a diverse germplasm base. Eleven separate batches containing 353 malt samples were mashed and wort SN levels determined using the Kjeldahl method were compared with the values predicted from the UV absorbance difference measurements using the calibration equation. The Kjeldahl method converts proteins and other wort nitrogenous compounds to ammonium salts which are then measured directly, whereas the UV absorbance response is due to the presence of peptide bonds only. Figure 3 shows a close linear relationship between the predicted and measured SN values with a correlation

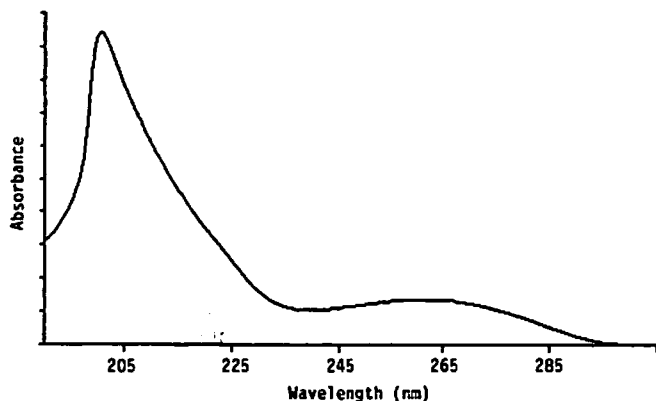


FIG. 1. Absorbance scan from 195 nm to 305 nm of wort diluted with 0.05% NaCl.

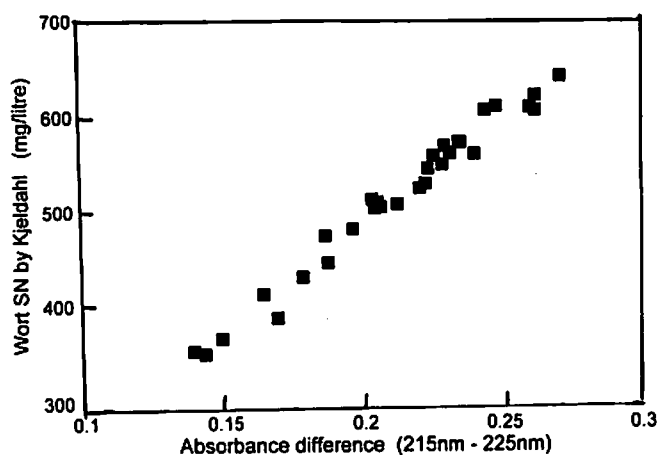


FIG. 2. Calibration data set for predicting wort SN comprising 31 barley samples each malted in duplicate and analysed by the Kjeldahl and spectrophotometric methods ( $r = 0.988^{***}$ ).

coefficient of  $r = 0.952^{***}$ . The regression equation relating these two data sets was:

$$\text{Wort SN (mg/litre)} = 2292 (A_{215}-A_{225}) + 22$$

This is similar to the calibration equation derived above and is also comparable to the relationship for 73 unhopped Congress worts reported by Franken-Luykx<sup>4</sup> below:

$$\text{Wort SN (mg/litre)} = 2321 (A_{215}-A_{225}) - 16 \text{ (where } r = 0.937\text{)}$$

The implications of the very good correlation between the UV and Kjeldahl data for a large number of genetically different malt samples are that either, the peptide proteins are by far the dominant form of soluble nitrogen compounds in the worts, or, that the proportions of peptide- to non-peptide nitrogen are generally constant in all wort samples.

The statistics for the data in Figure 3 are summarised in Table 1. There was very good agreement between the values obtained by both methods from the 353 wort samples. The standard deviation of the range of differences between values from the two methods was 19.57 mg/litre, equivalent to a coefficient of variation (CV) of 4.1% at the mean of the values measured (483.0 mg/litre). Both analytical procedures contribute errors of measurement to the relationship shown in Figure 3. For example, 10 replicate analyses of a single wort sample gave means and standard deviations of 0.285 ( $\pm 0.0034$ ) and 640.2 ( $\pm 6.96$ ) mg/litre for absorbance difference and SN

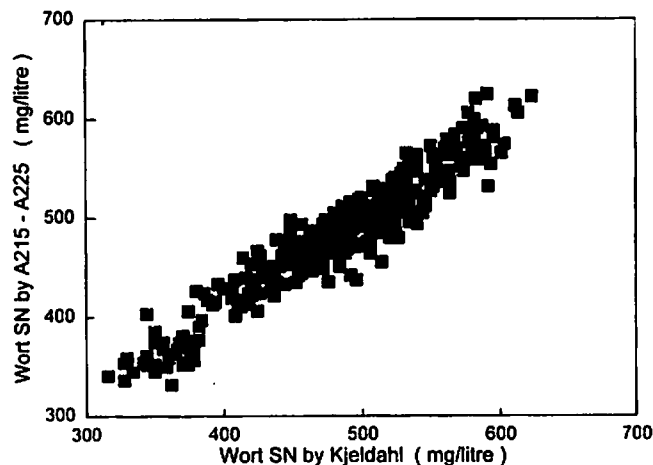


FIG. 3. Relationship between wort SN predicted by UV absorbance difference ( $A_{215}-A_{225}$ ) and wort SN measured by the Kjeldahl method. The correlation coefficient is  $r = 0.952^{***}$  ( $n = 353$ ).

TABLE 1 Measurement and prediction of wort soluble nitrogen in 353 micromalts

	$(A_{215}-A_{225})$	Kjeldahl N (mg/litre)	Predicted SN $(A_{215}-A_{225})^a$ (mg/litre)	Difference <sup>c</sup> (mg/litre)	Predicted SN $A_{215}^b$ (mg/litre)	Difference <sup>c</sup> (mg/litre)
Mean	0.2014	483.6	483.0	$0.6 \pm 19.57^d$	494.1	$-10.5 \pm 19.23^d$
Minimum	0.134	316	331		343	
Maximum	0.264	624	624		631	

<sup>a</sup>SN (mg/litre) =  $2249 (A_{215}-A_{225}) + 30$  ( $r = 0.988^{***}$ )

<sup>b</sup>SN (mg/litre) =  $1054 (A_{215}) - 25$  ( $r = 0.990^{***}$ )

<sup>c</sup>Difference between Kjeldahl and spectrophotometric methods.

<sup>d</sup>Standard deviation of the range of differences (c) between the Kjeldahl and spectrophotometric methods.

respectively, indicating similar CV's of 1.2% and 1.1% respectively for each procedure. The batch reproducibility was also comparable for each procedure. A standard check malt mashed in each of the 11 batches gave an absorbance difference mean of  $0.240 (\pm 0.0071)$  resulting in a mean prediction of  $569.0 (\pm 15.78)$  mg/litre of wort SN; and  $574.9 (\pm 16.80)$  mg/litre by Kjeldahl with respective CV's of 2.9 and 2.8%. Collaborative trials conducted by the EBC<sup>7</sup> and ASBC<sup>1</sup> to evaluate the spectrophotometric method produced comparable CV's relating to method repeatability which ranged from 1% to 5% depending on the SN level of test samples.

Waddell<sup>10</sup> reported that protein solutions at 215 nm obeyed Beer's law to an absorbance of up to 2.0. He suggested that measuring an absorbance difference ( $A_{215}-A_{225}$ ) or slope would restrict non-protein interferences found in human and animal plasma samples and noted that, although greater sensitivity could be obtained at wavelengths below 215 nm in the UV region, the results would be subject to light scattering problems. To test the feasibility of measuring wort SN at a single wavelength, absorbances were measured from a representative batch of 40 worts at 225 nm, 215 nm and also 201 nm, the absorption maximum for the peak shown in Figure 1. It was difficult to obtain stable absorbance readings from a spectrophotometer at 201 nm, although stability improved markedly at 2 nm on either side of this absorption peak. In contrast, it was noted that the absorbances at 225 nm and 215 nm of diluted worts were stable and that these samples could be stored overnight under ambient conditions without changing before reading in a spectrophotometer. Despite the low stability at 201 nm, it was found that there was a highly significant correlation between the difference ( $A_{215}-A_{225}$ ) and the measured absorbance at 201 nm ( $r = 0.964^{***}$ ). Indeed, the absorbances at 225 nm, 215 nm and 201 nm were all highly

correlated with each other ( $r$  values from  $0.970^{***}$  to  $0.990^{***}$ ).

Absorbance values at 215 nm and Kjeldahl data were used to derive a second calibration equation. There was found to be an excellent fit between these data ( $r = 0.990^{***}$ ), comparable with the ( $A_{215}-A_{225}$ ) equation (see footnotes in Table 1). Furthermore, when used to predict wort SN values from the validation set of 353 samples, the correlation between Kjeldahl and spectrophotometric SN values at 215 nm was  $r = 0.953^{***}$ , essentially the same as that from the absorbance difference data. Data shown in Figure 4 and Table 1, confirm that the two calibration equations were equally effective in predicting soluble nitrogen in wort. Using a single wavelength to measure wort SN does not seem to have been investigated previously. These results suggest that apparently for wort samples, there may be less interference in the low UV region from non-protein compounds than Waddell observed in mammalian tissues.

The adoption of a single wavelength measurement for predicting wort SN offers clear advantages over the absorbance difference method including speed, convenience, and simplicity. These factors are important when assessing the very large numbers of samples generated by breeding programmes. Measurement at 215 nm only, also avoids the problems of regularly re-zeroing a spectrophotometer at two wavelengths, and does away with errors accruing from measuring a difference between two similar numbers.

#### CONCLUSIONS

The application of a rapid, sensitive, and specific spectrophotometric method for determining SN in small wort samples produced from barley breeding programmes which offers comparable precision to the alternative Kjeldahl procedure is reported. The method is simple and avoids the safety and environmental problems associated with strong acids and aggressive and toxic reagents used with the Kjeldahl method. The stability of the absorbance readings of diluted wort samples for at least 16 hours also offers flexibility as well as rapidity to the analyst. The results demonstrate that the method is ideal for testing large numbers of breeders' lines and is particularly well suited to use with the test-tube scale mashing procedures now commonly used in this laboratory and elsewhere<sup>3,8</sup>, where the small volumes of wort recovered generally preclude the analysis of SN by the Kjeldahl method. In this instance, for the evaluation of most early- to middle-generation barley breeding stocks, the use of a single absorbance measurement at 215 nm provides a more convenient and attractive alternative to the absorbance difference procedure.

*Acknowledgements.* The authors thank Mrs Christine A Knights for skilled technical assistance and Mr D. B. Smith for helpful advice, and we acknowledge Dr P. I. Payne and Plant Breeding International-Cambridge for providing the facilities to carry out this work. One of us (RMH) gratefully acknowledges the award of a travel Fellowship from the New Zealand Institute for Crop & Food Research Ltd.

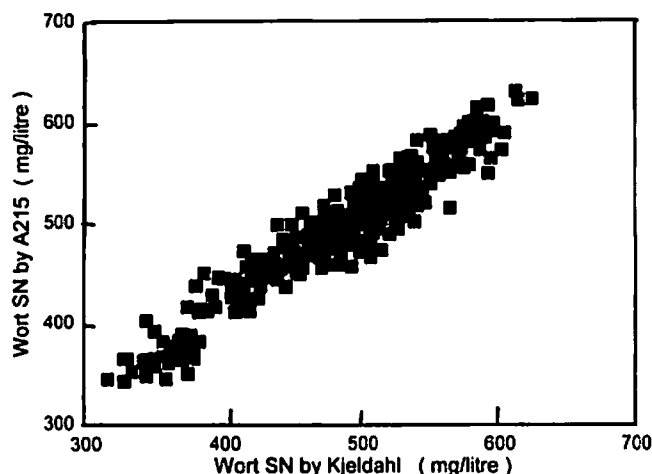


FIG. 4. Relationship between wort SN predicted by UV absorbance at a single wavelength ( $A_{215}$ ) and wort SN measured by the Kjeldahl method. The correlation coefficient is  $r = 0.953^{***}$  ( $n = 353$ ).

## REFERENCES

1. *American Society of Brewing Chemists Journal*, 1990, 48, 149.
2. Buckee, G. K. *Journal of the Institute of Brewing*, 1994, 100, 57.
3. Fox, G. P. & Henry, R. J. *Journal of the Institute of Brewing*, 1993, 99, 73.
4. Franken-Luykx, J. M. M. *Journal of the Institute of Brewing*, 1967, 73, 187.
5. Gothard, P. G., Morgan, A. G. & Smith, D. B. *Journal of the Institute of Brewing*, 1980, 86, 69.
6. Institute of Brewing, *Recommended Methods of Analysis*, 1991.
7. Lie, S. EBC collaborative trial. *Journal of the Institute of Brewing*, 1994, 100, 319.
8. Slack, C. R., Hancock, D. A., Haslemore, R. M. & Tunnicliffe, C. G. *Journal of the Institute of Brewing*, 1986, 92, 262.
9. Starr, C. & Smith, D. B. *Journal of Agricultural Science, Cambridge*, 1978, 91, 639.
10. Waddell, W. J. *Journal of Laboratory and Clinical Medicine*, 1956, 48, 311.