HGCA

PROJECT REPORT No. 98

THE DEVELOPMENT OF ROUTINE ASSAYS FOR WATER UPTAKE IN BARLEY

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THE DEVELOPMENT OF ROUTINE ASSAYS FOR WATER UPTAKE IN BARLEY

by

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CONTENTS

OBJECTIVES

The project aim was to devise simple, widely applicable procedures for measuring the distribution of water in barley endosperm. Benefits to maltsters would be optimization of malt quality, reduced malting loss, more effective water usage and facilitation of fine tuning of initial steeps. Methods could also be used widely for preliminary screening of barleys prior to malting to predict the most appropriate steeping conditions.

SUMMARY

Seven techniques were assessed to determine their potential for rapid measurement of water distribution through barley grains. Four were based on iodine vapour staining and two on the use of manganese as a tracer ion for water, both methods being originally developed under a previous HGCA-funded project (0031/1/87) at BRF International with the aim here being to adapt them into a simple and rapid form.

Insufficient iodine can be extracted from iodine vapour-stained grains for detection by titration, spectrophotometry or an iodide-specific electrode.

The rise in temperature of samples heated in a microwave oven has been ruled out as a method for determining moisture in the small sections of endosperm required for this work. This is due to poor repeatability.

A comparison of X-ray microanalysis (XRMA) data (The BRFI Malting Index) with a SEESCAN image analysis system or fibre optic probes from KIRSTOL in reflectance or transflectance modes, has shown potential for assessing water distribution in iodine vapour-stained samples. The image analysis does, however, require substantial manual input.

Manganese distribution, following steeping in manganese chloride, can be measured using the fluorescent marker hydroxynaphthaldehydethiosemicarbazone (HNTS). The fluorescent tag is sensitive to very low levels of manganese. The amount of manganese in aqueous extracts of steeped grains can be measured quantitatively. Further development may allow manganese distribution to be mapped and/or quantified in grain sections by epi-fluorescence on a light microscope.

1. INTRODUCTION

A technique, developed at BRF International under a previous project funded by the HGCA (0031/1/87; Baxter *et al* 1990), showed how iodine vapour staining could be used to trace water movement in barley endosperm. This work has been used to predict the malting quality of small samples of barley cultivars prior to assessment by the Institute of Brewing (Davies, 1992). The technique uses x-ray microanalysis (XRMA) on a scanning electron microscope to detect iodine levels, or alternatively, to measure levels of the tracer ion, manganese, that moves in with the water (Davies, 1991). It is desirable, however, to simplify this technique to make it more widely available, rapid and less expensive. This would allow non-specialized laboratories to make assessments on individual samples prior to malting.

2. MATERIALS AND METHODS

2.1 MATERIALS

Barley for the initial phase of this project were from the BRF International bulk stocks of Triumph, Blenheim or Puffin. The samples were screened by sieving and had grain widths in the range 2.5 - 2.8mm. This eliminated the possibility of variation in the rate of water uptake by grains smaller than 2.5mm (Zila *et al*, 1942).

Ten additional samples, for collaborative work with Scottish Crops Research Institute (SCRI) and also with Seescan Analytical Services, were selected from NIAB trial samples. The NIAB malting grades varied from 2 (feed) to 9 (malting).

Steeping was generally carried out at 16°C, using either 350g barley in a sweet jar or 2kg of barley in the BRFI small scale malting apparatus.

2.2 METHODS

2.2.1 Iodine Vapour Staining (Davies, 1991, 1992)

Grains were sectioned and put into a petri dish containing saturated iodine vapour created by a few flakes of iodine. After 60s exposure, the grains were removed. Only the hydrated areas of endosperm react with the iodine vapour and stain purple.

2.2.2 Release of Iodine from Vapour-Stained Grains

Iodine is a volatile halogen and it was therefore decided to try to release it from stained grains by heating. The released vapour was trapped in small quantities of water, hexane, ethanol or propan-2-ol and concentrated by evaporation.

2.2.3 Iodine Titration and Ion Specific Electrode

The titration is based on the reaction

$$I_2 + 2S_2O_3^{2-} \dashrightarrow 2I^- + S_4O_6^{2-}$$

A 1% (w/v) starch solution was used to indicate the progress of the reaction and hence the end point of the titration. With I_2 the liquid was blue and became colourless as the thiosulphate reduced it to iodide. The reaction has also been monitored by measuring absorbance in a spectrophotometer at 670nm.

An ion specific electrode for iodide has also been used to measure released iodine. The iodine was converted to iodide by using magnesium turnings.

For each extraction 50 grains were stained because greater numbers would render this technique too time consuming.

2.2.4 Fibre Optic Probes

Grains stained as in 2.2.1 were imaged using two fibre optic probe systems loaned by Kirstol, Manchester (from their Intellics range) operating in transflectance or reflectance modes. These probes were used to assess the value of colour or image intensity of the stained area as a way of quantifying iodine vapour staining and hence hydration.

2.2.5 Image analysis

An analysis system based on image capture by CCD (charge-coupled device) camera was borrowed from SEESCAN Analytical Services Ltd., Cambridge. Grains stained as in 2.2.1 were imaged on this system. Stained areas to be analyzed were manually delimited using a mouse. The software then calculated various parameters of the image area and measured the reflected light intensity. Image intensity was inversely proportional to the degree of staining.

2.2.6 Microwave Heating

A technique has been developed at BRFI by Nye *et al* (1992) that measures the temperature rise in a sample of barley or malt when heated by microwaves for

a set time. The basis of the technique has been established for other grains (Nelson & Kraszewski, 1990; Zhang & Brusewitz, 1991). The rise in temperature has been shown to be linearly related to the sample moisture content. Whilst the ultimate aim is to measure hydration in small parts of the endosperm, initial studies were targeted on determining the accuracy of the method. A related technique that measures the absorption of microwaves by a sample (Nelson & Kraszewski, 1990) is already used in the coal and paper industries and in the hop industry (Ganzlin & Söder, 1978).

2.2.7 Grain Milling Energy

Grain Milling Energy (Allison, 1989) was measured at the Scottish Crop Research Institute (SCRI, Invergowrie) using a "Comparamill". This is a modified hammer mill connected to a flywheel which slows down when samples are ground. Grains with a high milling energy cause the flywheel to slow down more quickly and the retarding energy imparted to the flywheel is expressed in Joule.

2.2.8 Fluorescence Studies Using Manganese as a Tracer Ion

Baxter *et al* (1990) showed how manganese could be used as a tracer ion for water movement when detected by XRMA in the scanning electron microscope.

A fluorescent label for manganese can be prepared by the following reaction at pH 3.5:

Hydroxynaphthaldehyde + Thiosemicarbazide → HNTS

The compound is purified by successive re-crystallisation in a solvent mix of water:acetone:ethanol (1:1:1; v/v).

When HNTS is added to manganese solutions and excited by light at 390nm the solution fluoresces at 450nm. Fluorescence was measured in a scanning fluorimeter. This characteristic has been used to measure manganese concentrations in aqueous extracts of grains steeped in up to 10% (w/v) manganese chloride solutions. It has also been applied to fluorescence of half grains steeped in the same solutions, sectioned and viewed under epifluorescence using a light microscope.

3. RESULTS AND DISCUSSION

3.1 Iodine Vapour Staining and Extraction

The amount of iodine staining in the endosperm is an indication of the degree of hydration because the purple stain produced is proportional to the number of iodine atoms which have become intercalated into the helix of hydrated amylose within the starch granule (Saenger, 1984). It has also been shown that it is easy to volatilize iodine by heating stained grains. The amount recovered in a water trap was, however, very small. It was not possible to improve the recovery of volatilized iodine by evaporative concentration or by using either hexane, ethanol or propan-2-ol.

The blue-black colour of the iodine-starch complex, formed by mixing iodine solution with soluble starch, was only produced above 2.5×10^{-5}M iodine (Figure 1). Thus end-point titration could not be used to detect concentrations lower than this and complex formation could not be detected any more readily using spectrophotometry. The small sample size of just 50 grains also meant that the amount of iodine recovered by volatilization and trapping was too small to be detected by either method.

Released iodine was converted to iodide using manganese turnings, which allowed iodide in sufficient quantities to be detected using an ion specific electrode. Iodide specific electrodes are claimed to have a detection minimum of about 5×10^{-8}M, but in practice, even when stable, the best maximum sensitivity was found to be 10^{-7}M. The levels of released iodide in aqueous extracts of barley were below this and hence could not be detected.

3.2 Fibre Optic Probe Analysis of Grains Stained with Iodine Vapour

Fibre optic probes from Kirstol were operated in transflectance or reflectance modes. The first instrument used two probes: one to illuminate and the other to detect the transflected light at an appropriate angle. The second instrument used just one probe, which illuminated the sample and also measured the light reflected back from the grain.

Table 1 shows how each probe ranked iodine vapour stained grains relative to XRMA data. Reflectance probe values are expressed relative to the corresponding unstained control at a given time. Transflectance data assigns a value of 100.0 to an unstained control grain at 0h steep. Both probes are able to distinguish between these grains and quantify the clear visual differences in staining.

It is also desirable to distinguish between varieties at a given point in the steeping schedule. Using the reflectance probe a set of 9 barleys of malting qualities ranging from NIAB grade 9 to 3 were investigated. Small samples were steeped for 3h at 20°C then sectioned and stained by the iodine vapour method. XRMA data was obtained for comparison. Table 2 shows that the reflectance probe data was less well spread than the XRMA data and only differentiated samples into three broad groups. These groups, however, showed little relationship to the ranking based on XRMA data. It should, however, be emphasised that some of these barleys were very close in malting quality and it might have been expected that the differences between some samples would be small.

3.3 Image analysis of iodine vapour stained grains

Experiments described in 3.2 were repeated with the Seescan image analysis system. The repeatability of the equipment was tested using unexposed photographic paper (Ilford Multigrade III), fixed in Ilford "Ilfospeed" 1:4 dilution. The intensity of ten small areas showed excellent repeatability.

The repeatability of measurement on the same grain was also found to be good. Ten sequential measurements were made on one vapour-stained grain of Derkado (Table 3). The variation in delimited area emphasises the subjective nature of this manual system.

A comparison was made between water distribution in good and poor malting varieties after a 6h steep (Table 4). Ten areas of the endosperm of either Dove (grade 2) or Derkado (grade 9) were measured. Whilst there was some overlap in the degree of staining (intensity value) in some areas, the overall average intensity was less (and hence hydration was better) in the malting grade barley. Again this data showed a significant variation in the delimited area despite having samples screened between 2.5 and 2.8mm.

A more comprehensive range of barleys was screened to examine the possibility of using this system to differentiate malting varieties. Table 5 shows that there was poor agreement between malting grade and image intensity. This was most probably due to differences in colour of the grain and possibly due to uneven staining.

Water distribution as steeping progresses was also assessed in Derkado. Figure 2 shows averaged data from seven steeps. Image intensity dropped, hence staining and hydration increased during steeping, as expected. Individual sets of data, however, were quite variable for different steeps (Figure 3). The implication is that a larger number of samples is required for reliable results.

3.4 Microwave Heating

This technique has been reported to have the advantage of measuring the amount of water in a small sample of barley (15g) directly (Nye *et al*, 1992). The sample is accurately weighed ($\pm$0.2g) into plastic tubes of specified dimension and sealed with a screw top which has a small (2mm) hole drilled in it. The initial grain temperature is measured and the tube put into a microwave oven. To prevent too high a temperature rise, a small beaker of water is also put into the microwave to act as a buffer for a high proportion of the microwaves. The sample is irradiated for 30 seconds and the temperature measured again. A standard curve can be constructed using samples of known moisture content determined by oven drying. The rise in temperature (ΔT) is linearly related to the moisture content. The slope of this rise is found to change at about 11% moisture, thus measurements of moisture in the range 10-12% may be prone to greater error unless measurements are very accurate.

Using steeped samples, with moisture contents of 30% or more, however, the temperature rises were subject to variation of up to $\pm$ 5°C between samples from the same batch (results not shown). The aim, therefore, has been to find a set of conditions that would produce a rise in temperature of the sample with an error limit

of $\pm$ 1 °C.

At the higher moistures found in steeped samples a much larger ΔT was measured than in the original study. The maximum temperature achieved by these samples also fell rapidly when they were moved to ambient conditions to determine ΔT. Reducing the time in the microwave has been shown to result in a smaller ΔT and has made measurement of the maximum sample temperature more accurate. Adjusting the volume of the water which is used to buffer the microwave irradiation and changing it for every determination of ΔT, together with pre-attemperation of grain samples to about 21 °C, has also been shown to improve the accuracy of the method.

The technique is useful for determining moisture in barley and malt. Figure 4 shows data obtained from samples taken during drying of malt from 15% to 5%. The average values obtained for each sample follow the expected pattern of drying. There is evidence for a flexion point at 11% but within each set a considerable degree of error was found.

The microwave heating technique has been successfully developed for measuring moisture in bulk barley samples. To produce information on hydration similar to the XRMA / iodine vapour method, however, small sections of the endosperm need to be excised from the grain before heating and determination of ΔT. It is considered that the accuracy and repeatability of the technique is not sufficient to measure hydration of such samples where there may be only very small differences in moisture content.

3.5 Grain Milling Energy (GME)

NIAB barley samples were used to compare the BRFI Malting Index with GME (Table 6). Fourteen samples were steeped for 6h then air-rested prior to iodine vapour staining and measurement by XRMA. The Grain Milling Energy was determined for a second set of the fourteen samples. The rank is ordinal and does not refer to the malting grade. The samples are represented by intake numbers. The right hand column shows how the samples were ranked according to NIAB grade. The BRFI Malting Index showed a good correlation with both NIAB grades and GME. There were some anomalies; for example sample 217 was ranked much higher by GME than by the other techniques and sample 207 was ranked differently by each technique. The feed varieties, however, were ranked appropriately by all three methods. If the slight anomalies are ignored then both existing tests are shown to be reliable predictors of malting quality and are useful standards by which to judge newer and simpler techniques.

3.6 Fluorescence Studies with Manganese

The compound hydroxynaphthaldehydethiosemicarbazone (HNTS) has a base level of fluorescence when pure and dissolved in ethanol. Manganese enhances the fluorescent yield substantially (Perez-Bendito *et al,* 1984). Figure 5 shows how this base level of fluorescence increased with incubation time. Addition of 30ng of manganese greatly enhanced fluorescence and even 3ng had some effect. Thus the technique is highly sensitive to very low levels of manganese in solution.

Interference from other ions that might be present in barley was also tested. Addition of 30ng of different ionic solution was made to a vial containing HNTS (Figure 6). All additions, with the exception of sodium, reduced the base fluorescence slightly. Even though sodium increased fluorescence it was still only to about a third the level of manganese. Since the levels of sodium in barley are negligible it should not affect the technique.

The technique of introducing manganese chloride in steep was developed using 10% (w/v) solutions. These have the effect of reducing malt development when used throughout steeping. The high sensitivity of this technique means that less manganese needs to be present in the grain to be detected. Figure 7 shows that significant levels of fluorescence can be produced from aqueous extracts of barley steeped in just 2% (w/v) manganese chloride. In fact the original 10% steeping solution creates very high levels of fluorescence. Note that in this figure the scale is in thousands whereas in the other Figures the scale is up to 1000. A reading of 1000 is sufficient to obtain accurate results.

Addition of HNTS to sections of barley grains to enable viewing by epifluorescence has proved troublesome. Autofluorescence in barley and sample thickness both contribute to a poor fluorescent yield. This aspect of the technique poses considerable technical difficulties.

4. CONCLUSION

Fibre optic probes used in conjunction with iodine vapour staining showed promise for the quantification of hydration during steeping. Image analysis too was able to broadly categorize good and poor malting varieties. Both techniques suffer from the usual difficulties inherent to imaging: specimen surface roughness and uneven illumination. Semi-manual systems were used in this work since a routine assay would require a cheap system. More expensive and automatic systems generally offer more sophisticated image processing whilst retaining the initial image capture method.

Measurement of iodine extracted from stained grains is not feasible. The rise of temperature during microwave heating is impractical due to poor repeatability and sample preparation is too time consuming for this application.

The most promising development is the use of HNTS as a fluorescent tag for manganese. Previous work required 10% (w/v) manganese chloride in the steep liquor to allow detection by XRMA. Aqueous extraction of manganese from grains steeped in manganese chloride has shown that a level of less than 1% in steep can be detected by virtue of the high sensitivity of this assay system. It would be useful if a simple illumination and viewing system could be developed to allow HNTS to be applied directly to grain sections and viewed in a system similar to that used in the Calcofluor test. In this project, viewing grain sections by epifluorescence under a light microscope has shown a low fluorescent yield. If this can be overcome the HNTS technique could provide a simple alternative assay for viewing and possibly quantifying barley hydration.

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TABLE LEGENDS

Table 1: **Use of Fibre Optic Probes to Assess the Hydration of Iodine Vapour-Stained Barley During Steeping**

(A comparison with XRMA data)

Two systems supplied by Kirstol (Intellics range) were used to quantify iodine staining in steeped grains. The reflectance system used a single fibre optic cable to both illuminate the sample and collect reflected light. The transflectance system used two fibre optic probes. One probe illuminated the sample and the second measured transflected light. Both systems were able to quantify staining and hence differences in hydration in steeped grains.

Table 2: **Use of Fibre Optic Probes to Assess the Malting Grade of Iodine-Vapour Stained Barley**

Barleys of various malting grade were steeped for 3h at 20°C. A reflectance probe measured staining but could only separate the samples into three broad groups. XRMA data showed a better separation.

Table 3: **Use of Image Analysis to Measure Endosperm Area and Intensity of Iodine Staining in Barley.**

Staining of a single grain of variety Derkado was assessed ten times. Areas to be measured were delimited manually using image analysis software supplied by SEESCAN. There was considerable variation in the delimited areas. Staining intensity was, however, quite consistent.

Table 4: **Use of Image Analysis to Distinguish Between Malting and Feed Barley Varieties.**

Staining intensity was measured using the SEESCAN system for two barleys with widely different malting grades: Derkado (Grade 9, Malting) and Dove (Grade 2, Feed). Averaged results show a significant difference in intensity between the two samples, but there is considerable variation in the individual data sets.

Table 5: **Use of Image Analysis to Rank Barley Varieties After Iodine Vapour Staining.**

Using eleven varieties of barley with different malting grades it was not possible to obtain a clear progression of image intensities from malting to feed grade.

Table 6: **Comparison of the BRFI Malting Index and Grain Milling Energy as Indicators of Malting Quality.**

Fourteen samples were steeped for 6h then air-rested prior to iodine vapour staining and measurement by XRMA. The Grain Milling Energy was determined for a second set of the fourteen samples. In general there was good agreement between the two techniques in the ranking of barley samples. The agreement was particularly good for feed varieties.

FIGURE LEGENDS

Figure 1: **The Sensitivity of the Reaction Between Iodine and Soluble Starch.**

The absorbance of the starch-iodine complex was measured in a spectrophotometer (670nm) at different iodine concentrations.

Figure 2: **Reflected Light Intensity as a Measure of Hydration During Steeping**

Data from a Seescan image analysis system using Derkado barley. Results are the average of seven steeps.

Figure 3: **Reflected Light Intensity as a Measure of Hydration During Steeping - Individual Steep Data**

There was considerable variation in the image intensity profiles for individual steeps. This emphasizes the need for multiple data sets for best accuracy.

Figure 4: **Use of Microwave Heating to Measure Grain Moisture Content**

Samples were taken from barley in the process of drying from 15% to 5%. There is evidence of a flexion point at 11%.

Figure 5: **Effect of Time on HNTS Fluorescence**

HNTS fluorescence *in vitro* increases with storage time. Addition of small amounts of manganese greatly enhances fluorescent yield. Samples incubated in cuvettes at room temperature.

Figure 6: Relative Fluorescence of HNTS-Ion Complexes

Addition of ions to HNTS solutions generally reduces fluorescence slightly. Sodium increases fluorescence to above one third the levels when manganese is added. The levels of sodium in barley are low thus the assay should not be affected.

Figure 7: Fluorescence of Barley Extracts From Grains Steeped in Manganese Chloride

Low levels of manganese in steep liquor can be detected in aqueous extracts of barley during steeping. Samples are ground in water and the solution filtered. The extract fluorescence is measured on a scanning fluorimeter at 450nm.

Table 1:
Use of Fibre Optic Probes to Assess the Hydration of Iodine Vapour-Stained Barley During Steeping

A Comparison With X-ray Microanalysis Data (XRMA)

Sample Type	XRMA data (P-B)	Error range	Reflectance Probe	Transflectance Probe (*)
0h steep control	0	± 0		
0h steep I_2 stained	0	± 0	0	76.8
2h steep control	0	± 0		
2h steep - I_2 stained	342	± 86	7	52.2
6h steep control	0	± 0		
6h steep - I_2 stained	1675	± 200	15	45
6h steep, 18h air rest-control	0	± 0		
6h steep, 18h air rest- I_2 stained	3782	± 500	60	24

(*) An unstained control grain was assigned a reference value of 100.0 **Barley Variety: Blenheim**

Table 2:
Use of Fibre Optic Probes to Assess the Malting Grade of Iodine Vapour Stained Barley

A Comparison With X-ray Microanalysis Data (XRMA)

Ordinal Rank (Not NIAB Grade)	XRMA data (P-B)	Reflectance Probe
1	211	240, 234, 211, 230
2	208	
3	230, 241	
4		
5	239	208
6	240	241, 239, 223, 226
7	223, 226	
8		
9	234	

Samples steeped for 3h then stained with Iodine vapour Numbers represent sample codes

Table 3:
Use of Image Analysis to Measure Endosperm Area and Intensity of Iodine Staining in Barley

Using a Single Grain of the Variety Derkado

Area of Endosperm	Intensity of Staining
6.515	245.1
6.272	246.1
6.115	246.4
6.040	246.7
6.069	246.7
6.173	246.3
6.183	246.6
5.880	246.8
5.986	246.7
6.025	246.7
Average	**6.126**	**246.4**
S.D.	**0.493**	**0.5**

Table 4: Use of Image Analysis to Distinguish Between Malting and Feed Barley Varieties

Derkado (Grade 9)

Area	Intensity
8.207	93.5
8.886	138.4
8.533	62.44
8.212	102.2
10.12	88.62
8.193	39.68
8.266	126.3
12.79	59.1
8.238	70.25
7.362	80.34

Dove (Grade 2)

Area	Intensity
8.471	103.4
8.728	123.0
9.436	163.9
10.75	63.03
9.905	116.3
6.78	189.7
9.743	140.8
8.102	102.8
10.33	82.6
9.573	76.6

Average	**8.881**	**86.09**	**9.182**	**116.2**
S.D.	**1.54**	**30.55**	**1.178**	**39.7**

Table 5: Use of Image Analysis to Rank Barley Varieties After Iodine Vapour Staining

Variety	NIAB Grade	Image Intensity
Derkado	9	86.09
Chariot	9	146.18
Dallas	9	97.77
Blenheim	8	118.4
Triumph	8	120.36
Nomad	8	127.73
Decor	7	138.59
Chad	6	105.05
Hart	4	117.02
Dove	2	124.00
Shirley	2	118.53

Table 6:
Comparison of Grain Milling Energy and the BRFI Malting Index as Indicators of Malting Quality

Ordinal Ranking	BRFI Index	GME (Avr)	Suggested NIAB Rank
1	208	217	208
2	209	208	211
3	211, 215	209	209
4		211	213
5	213	216	219, 207
6	207	219	
7	219	213	217, 205
8	217	205	
9	205	206	216
10	216	215	215, 206
11	206	207	
12	214	214	214
13	212	212	212
14	218	218	218

Numbers refer to sample codes

Figure 1: The Sensitivity of the Reaction Between Iodine and Soluble Starch

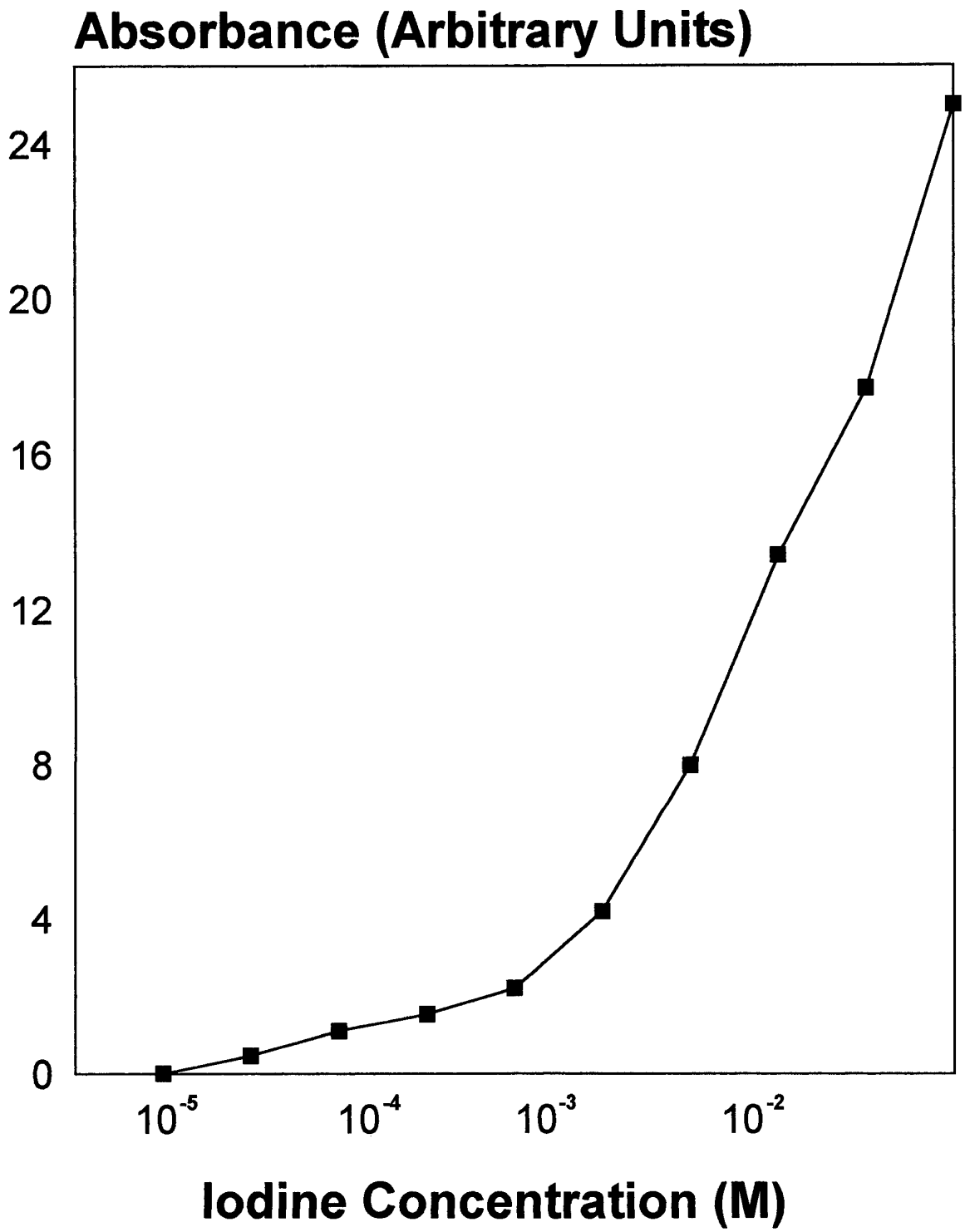


Figure 2: Reflected Light Intensity as a Measure of Hydration During Steeping

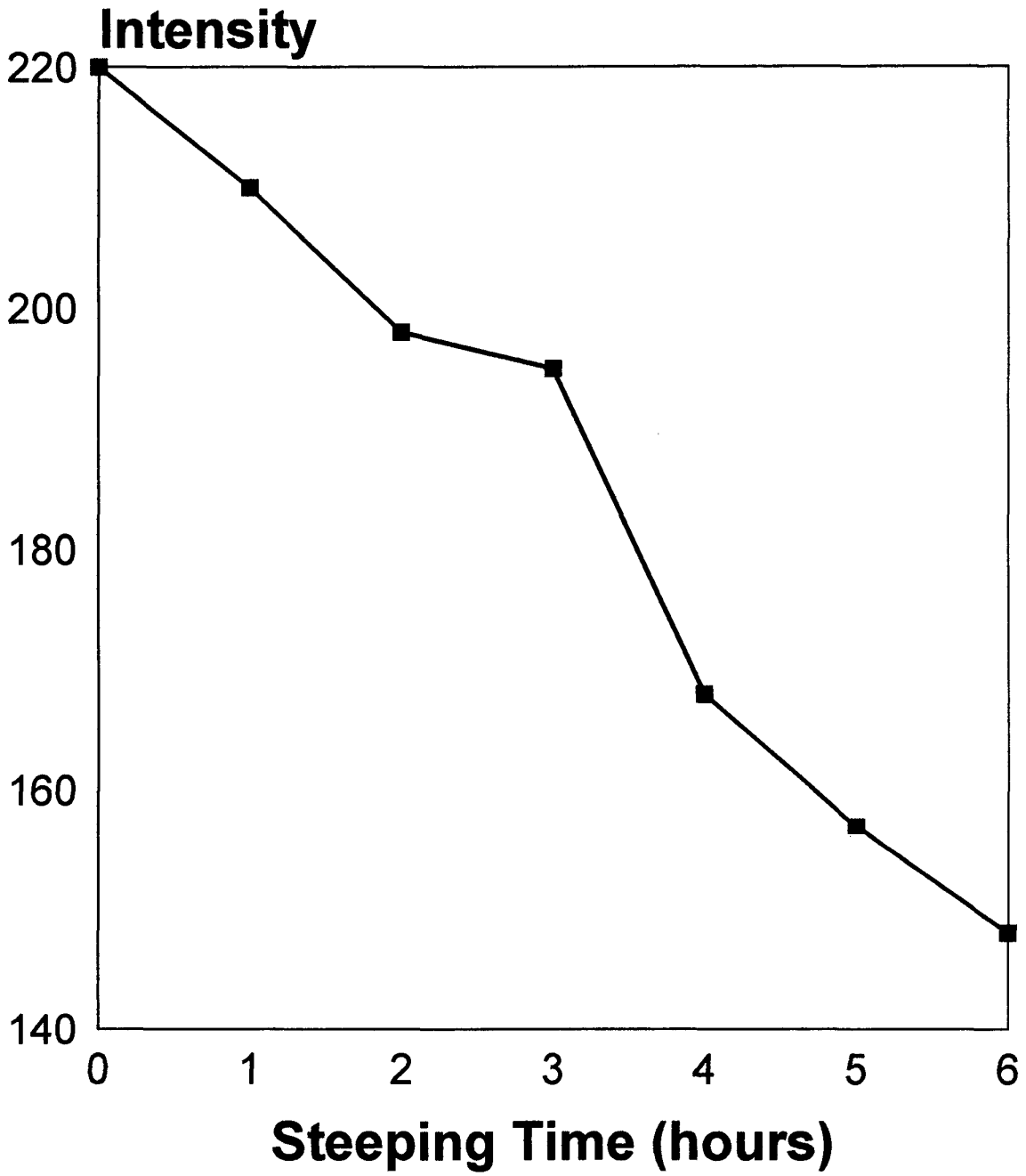
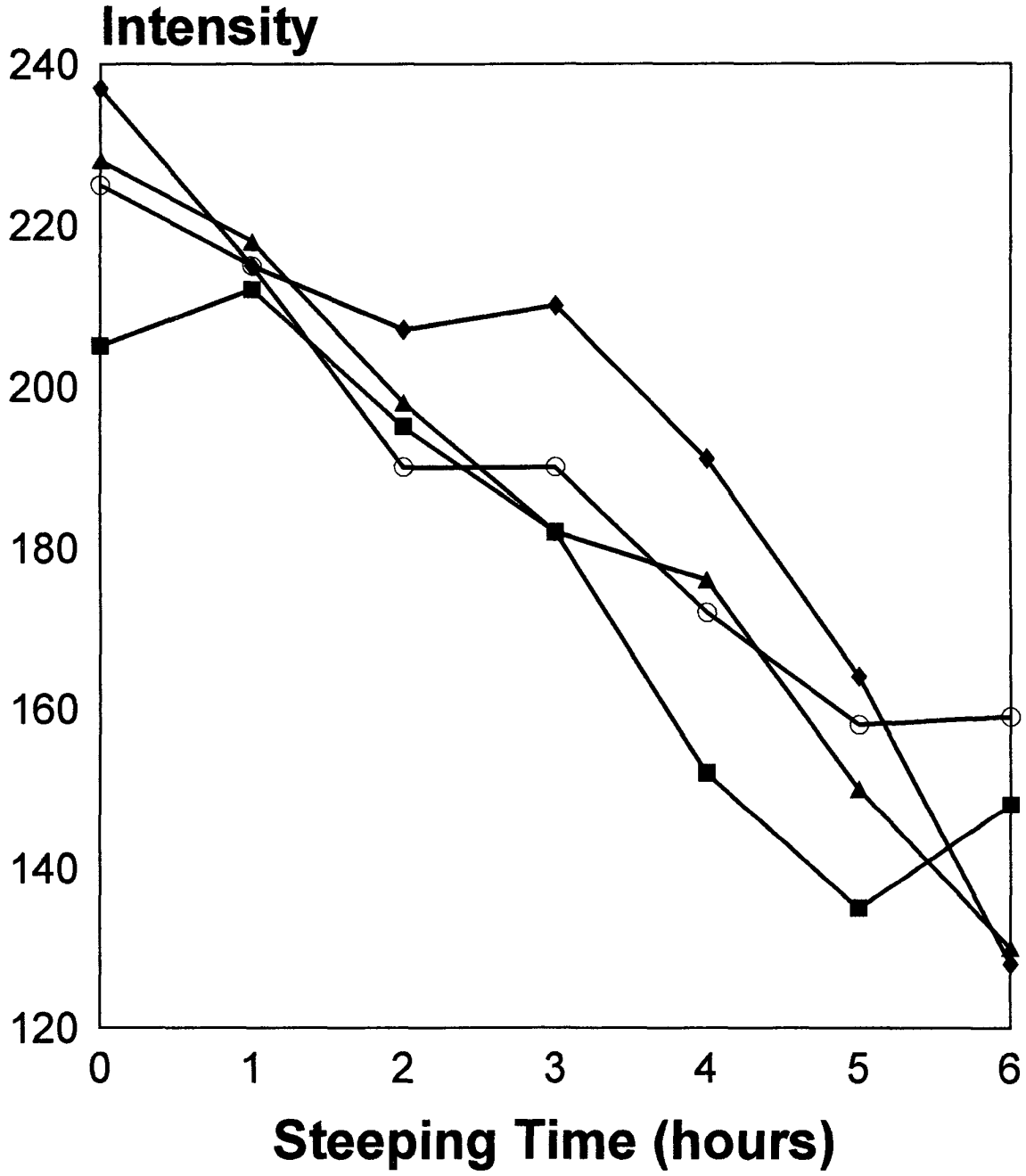


Figure 3: Reflected Light Intensity as a Measure of Hydration During Steeping

Individual Steep Data



Each line and symbol represents a different steep

Figure 4: Use of Microwave Heating To Measure Grain Moisture Content

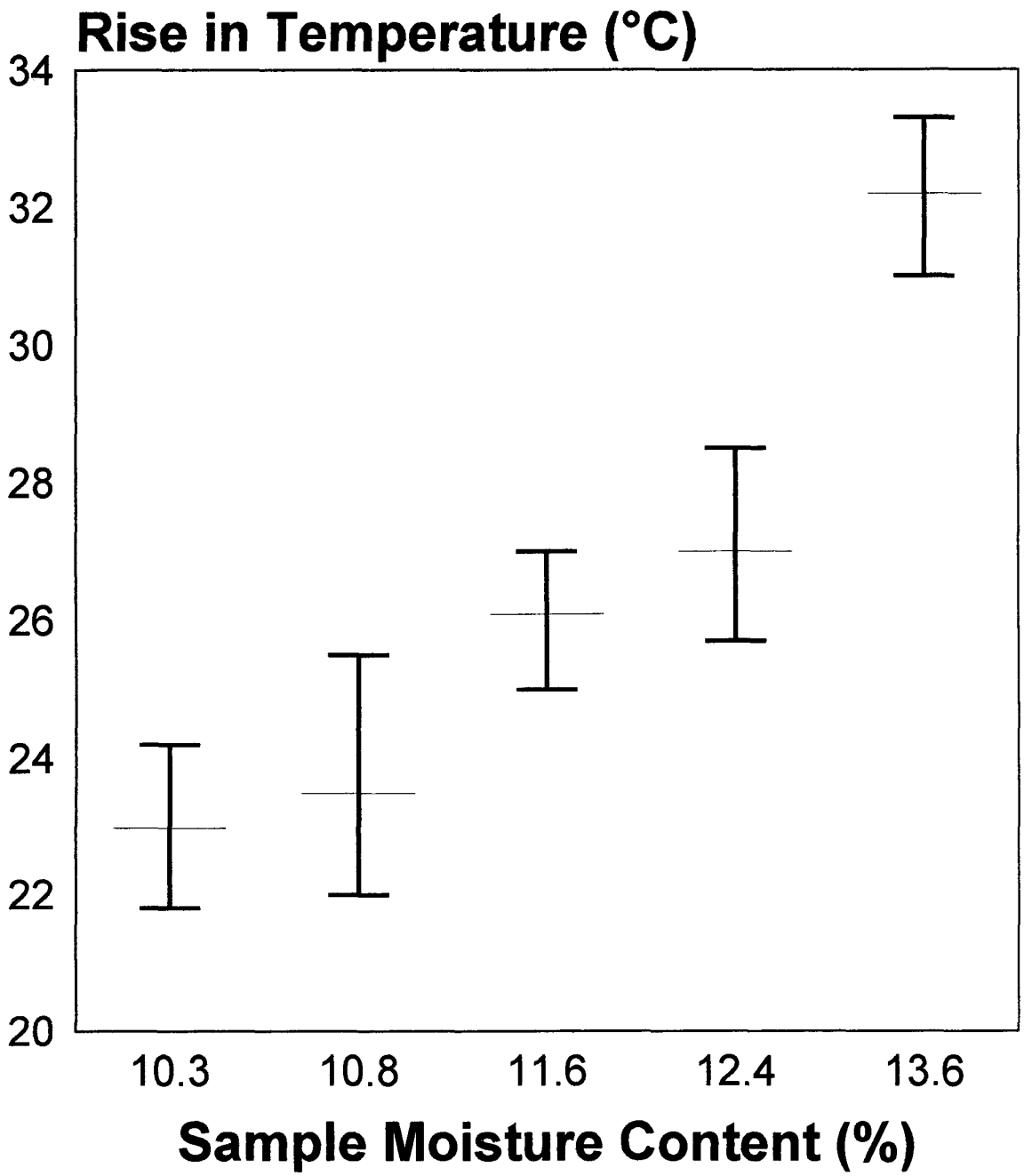


Figure 5: Effect of Time on HNTS Fluorescence

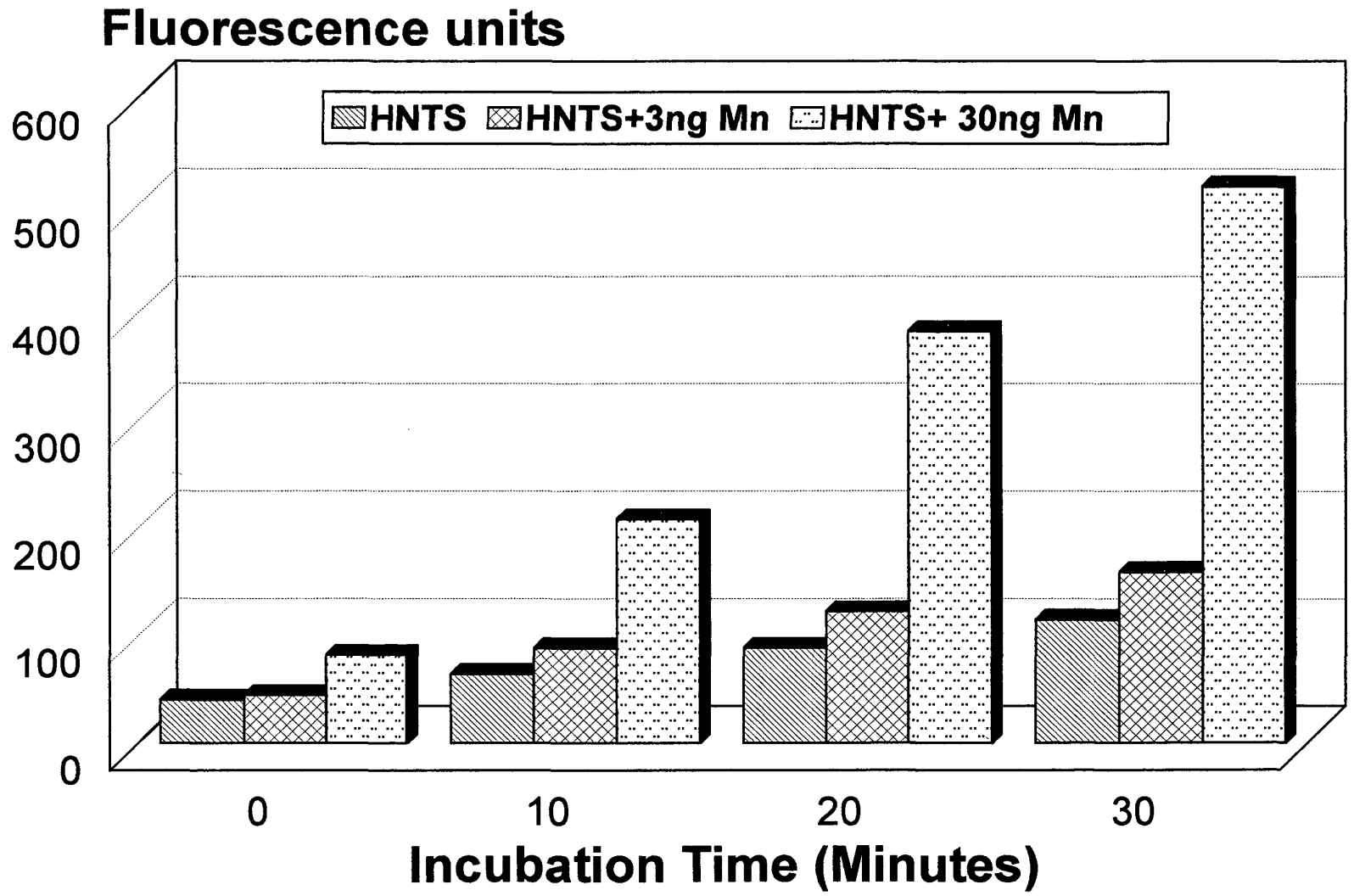


Figure 6: Relative Fluorescence of HNTS-Ion Complexes

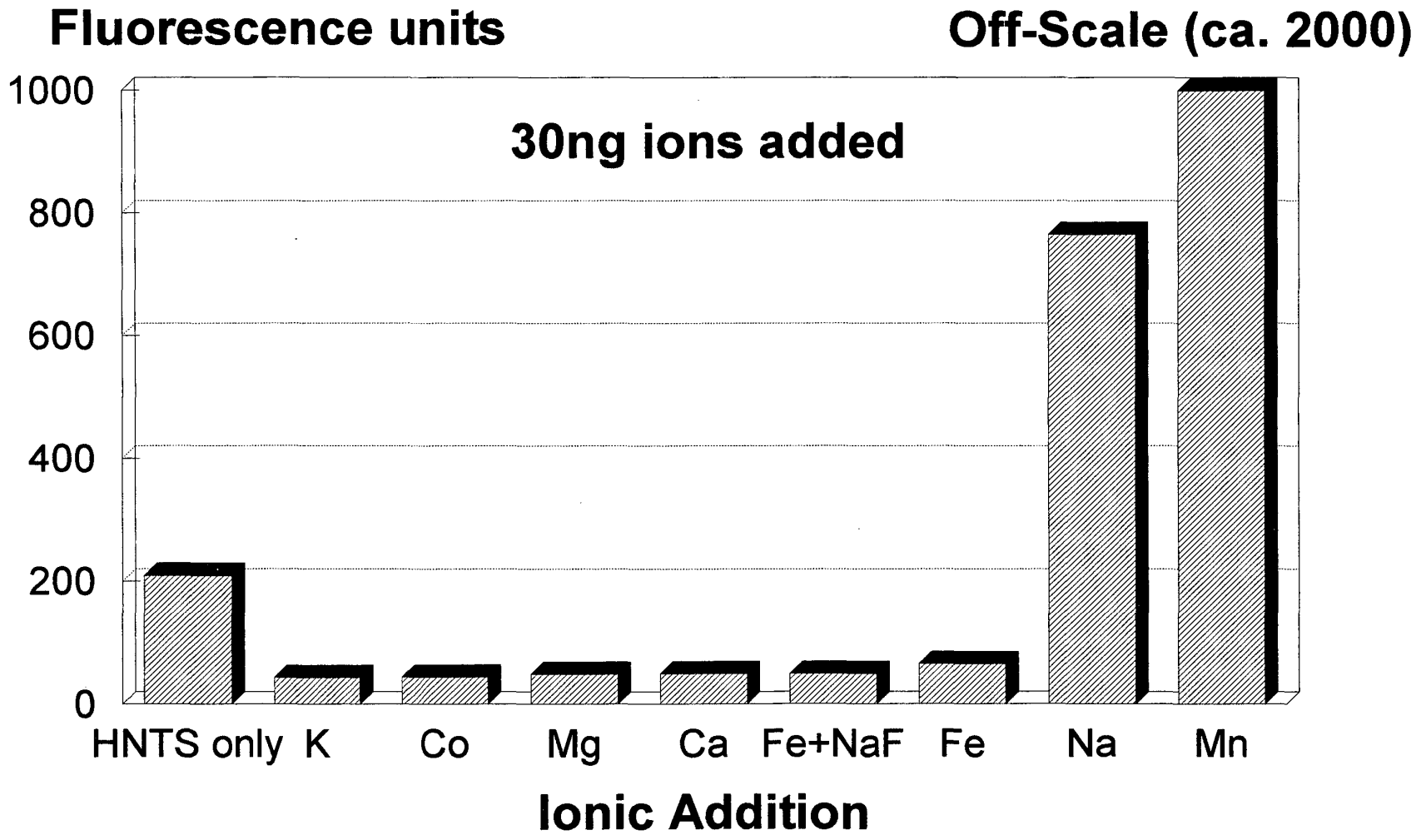


Figure 7: Fluorescence of Barley Extracts From Grains Steeped in Manganese Chloride