A STUDY OF THE SEPARATION OF STORAGE PROTEINS FOR VARIETAL IDENTIFICATION AND OF ß-GLUCAN AND ß-GLUCANASE LEVELS IN MALTING BARLEY

BY

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DEDICATION

To my family, especially my parents Elsie and Vincent for their tremendous encouragement and support

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DECLARATION

I declare that this thesis has not been submitted as an exercise for a degree at any other university and is entirely my own work, except where otherwise stated

CARINA F. FITZPATRICK

Carina Fitzpatrick

March, 1995

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I want to thank most sincerely Professor Richard O'Kennedy and Dr James Burke for giving me the opportunity to do this research To my supervisors Professor Richard O'Kennedy, Dr Andreas Frohlich and Dr James Burke I wish to express my gratitude for their help, enthusiasm and guidance over the past four years

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Finally I would like to thank my mother, father and brother for their support and interest

ABBREVIATIONS

HPLC	High pressure liquid chromatography
RP	Reverse phase
IE	Ion exchange
TFA	Trifluoroacetic acid
DTT	Dithiothreitol
CAP	3-(cyclohexylammo)- propanesulphonic acid
DMSO	Dimethylsulphoxide
DEAE	Diethylaminoethyl cellulose
BSA	Bovine serum albumin
Gopod	Glucose oxidase-peroxidase
C18	Octadecylsilane bonded silica gel
C4	Butylsilane bonded silica gel
C3	Propylsilane bonded silica gel
N	Nitrogen
ha	Hectare
CV	Coefficient of variation
s d	Standard deviation

ABSTRACT

High performance liquid chromatography, using either reverse phase, anion exchange or chromatofocusing columns was used for the resolution of hordems extracted from Irish barley varieties The possibility of using the hordein elution profiles for the routine identification of Irish barley varieties was investigated

Extracted hordeins were well resolved with the two reverse phase columns used in the present work and the difference between the elution profiles of the thirteen varieties examined were very obvious It was possible to reduce hordein elution times for the reported 110 minutes to about 50 minutes without loss in resolution by using a C4 rather than C18 column As the hordein profiles were repeatable even after extended column use, they can be used for the positive identification of Irish barley varieties

Hordein elution profiles characteristics of a particular variety were also obtained with anion exchange column and chromatofocusing, but the resolution with both was much poorer than with reverse phase columns The repeatability of hordein profiles with anion exchange columns was poor, resolution deteriorated rapidly after five injections and elaborate column cleaning was required to restore it Repeatability was somewhat better with chromatofocusing but also a lengthy column cleaning was required after every ten injections For both columns hordein profiles obtained before and after column cleaning were different, hence the techniques in their present form cannot be used for varietal identification

The two most commonly used methods for the determination of ß-glucan levels namely the cellulase and the lichenase assay were evaluated Considerably higher total ß-glucan levels were obtained with the cellulase than with the lichenase assay but it was not possible to establish from the available data which was the more accurate method However, as the lichenase assay is the preferred method of the brewing industry it was used for estimating the effect of variety, agronomy and environmental factors on ß-glucan levels in barely Varietal effect on ß-glucan levels was evident, intervarietal differences were statistically significant and even if ß-glucan levels for all varieties changed with season, the order of values remained constant Autumn sowing dates had no effect on ß-glucan levels at two locations and the effect of applied nitrogen at three locations was inconclusive A strong environmental effect on ß-glucan levels was also noted, in that levels seem to be effected by soil moisture

during grain filling and decline with decreasing soil moisture

Barley samples used for the determination of external effects on ß-glucan levels were micromalted to evaluate external effects on ß-glucanase levels in malt The "dye- labelled ß-glucan assay" was used for the determination of ß-glucanase levels in these samples, because it was found to be the most precise of the three reported methods evaluated by an independent study The other two methods were viscometry and radial diffusion assay There was a definite varietal effect on ß-glucanase as the differences between levels corresponding to the six varieties were statistically significant and the order of values remained constant for two seasons The environmental effect was also shown by the fact that three varieties had consistently lower ß-glucanase levels in one of three locations, at different rates of applied nitrogen No explanation could be advanced for the observed effect Different autumn sowing dates had no obvious effect on ß-glucanase levels in malt

CHAPTER 1

IDENTIFICATION OF MALTING BARLEY VARIETIES BY HIGH PRESSURE LIQUID CHROMATOGRAPHY

1.1 INTRODUCTION

1.1.1 Methods of barley varietal identification

1.1.1a Visual methods and electrophoresis

Identification of barley cultivars is important for the determination of the suitability of the crop for a particular environment, or of grain for certain end uses - such as feed, food or malting. The grower needs to know his crop because cultivars differ in their yield, quality and disease resistance The manufacturer needs to know the identity of the barley grain, because only certain cultivars are suitable for malting or for food In addition, seed merchants are legally required to supply seeds of specified varietal purity It is highly desirable therefore to have a quick and reliable method for varietal identification

Traditional methods for the determination of varietal identity varied considerably from crop to crop, but they generally involved detailed morphological studies of plant characters These methods were time consuming and expensive as large areas were required to grow a suitable number of plants A simpler morphological method for the determination of barley varieties involved staining and microscopic examination of seeds The technique has been useful for the identification of older barley varieties, but most newer varieties are indistinguishable

Polyacrylamide gel electrophoresis is a more convenient technique for varietal identification than morphological characteristics as it relies on characteristic patterns generated by the resolution of prolamines (alcohol-soluble seed proteins) Since hordeins are polymorphic, that is their composition is linked to variety, electrophoretic band patterns can be used for barley varietal identification Extraction of hordeins from the grain or flour is very simple and a well resolved band pattern can be obtained within 24 hours Electrophoresis is far more reliable than the visual methods and it has been used successfully for the identification of certain barleys (Shewry *et al*, 1978a) However, not all varieties can be distinguished from each other Some Canadian barley varieties give identical hordein patterns (Marchylo *et al*, 1984) and only 32 different patterns could be obtained out of 160 European barley varieties (Allison and Bam, 1986) Another disadvantage of the technique is that a great deal of practical experience is necessary for the casting of uniform electrophoresis gels required for varietal identification

1.1.1b Reverse phase high pressure liquid chromatography

In addition to electrophoresis, RP-HPLC (reverse phase high pressure liquid chromatography) has been also used for varietal identification RP-HPLC is one of the more widely used techniques for the separation of biomolecules Reverse phase chromatographic columns are packed with a hydrophobic gel of 5 or 10 μ particle size, which consists of a large number of hydrocarbon chains (usually hydrocarbons from C3-C18) covalently bonded to silica gel Compounds are separated on basis of their hydrophobicity Most proteins, even those which are weakly hydrophobic, bind to the matrix and form a narrow band on the top of the column. Bio-molecules are selectively eluted from this band in order of increasing hydrophobicity by gradually increasing the organic solvent component (gradient elution) of the mobile phase Addition of ion pairing agents, such as trifluoroacetic acid or phosphoric acid generally improve peak shape and resolution of the eluted polypeptides

Early reverse phase materials with pore diameter below 100Å were only suitable for smaller bio-molecules, but since the availability of wide pore matrix of 300Å pore diameter, RP-HPLC can be used for the separation of large biomolecules such as proteins up to approximately 200,000 daltons The technique is particularly suitable for the separation of alcohol soluble storage proteins such as hordeins in barley and gliadins in wheat as these are hydrophobic with molecular weights ranging from about 30,000 to 80,000 daltons (Shewry et al , 1978b)

Hordeins for RP-HPLC are extracted from barley with aqueous alcohol containing 1-2% (w/v) reducing agents - to reduce cross-linking disulphide bonds (Marchylo *et al* , 1984) It is possible also to resolve proteins without reducing disulphide bonds (Wingad *et al* , 1986) but resolution is much poorer and some varieties appear to have identical hordein elution profiles The preferred mobile phase for the chromatography of hordein is aqueous acetonitrile with TFA as ion paring agents, and components are eluted by increasing gradually the concentration of organic solvent m the mobile phase

RP-HPLC has been used successfully for the identification of American (Bietz, 1983) and Canadian (Marchylo *et al* , 1988) wheat varieties and European (Allison and Bain, 1986) and Canadian (Marchylo and Kruger, 1984) barley varieties by using gliadin and hordein elution profiles The work on barley varietal identification indicated that RP-HPLC is a superior technique to the traditional electrophoresis for varietal

identification because it can distinguish between varieties with identical electrophoretic patterns (Allison and Bain, 1986) In addition, it is far more convenient for the routine varietal identification of bulk samples than electrophoresis, for sample preparation is minimal and the chromatographic process can be fully automated.

1.1.1c Ion exchange chromatography

While RP-HPLC was successful in distinguishing between many barley varieties with identical electrophoretic pattern (Allison and Bain, 1986) the technique is not without drawbacks In the case of some varieties the difference between elution profiles is very small (Allison and Bain, 1986) and a confirmatory evidence obtained by a different technique would be of advantage Furthermore reported analysis times (105-120 mins) are relatively long, and would make determination of varietal purity very time consuming Finally, poor elution profile reproducibility after repeated runs has been pointed out (Bietz 1985, Courage Ltd 1992)

The use of IE-HPLC (ion exchange high pressure liquid chromatography) for varietal identification has been recommended by Bietz (1985) on the basis that ion exchange columns should be more stable than reverse phase columns as they are more uniform chemically (polymers have a better defined chemical structure than bonded silica gels) and more selective as non-ionic materials are not absorbed by the matrix These factors should, at least in theory, improve the reproducibility and resolution of the obtained hordein elution profiles Furthermore, as only ionic components are absorbed, it should be possible to extend the life of the column indefinitely by regular washing with a strong counter-ion solution

Ion exchange resin separates biomolecules on the basis of charge interactions rather than by hydrophobicity The column matrix consists of organic resin beads with sulphonic acid or quaternary amine groups on the surface Proteins at certain pH values carry a net positive or negative charge, and when applied to a resin of opposite charge they form a narrow band at the top of the column Separation of the absorbed proteins takes place when they are displaced by a counter-ion according to the equilibrium

$$R^{\oplus}P^{\ominus} + C1^{\ominus} \rightarrow R^{\oplus}\,C1^{\ominus} + P^{\ominus}$$

where $R^{\oplus}$ is the resin $P^{\ominus}$ the protein and $C1^{\ominus}$ the counterion In the case of cation exchange resins the charges are reversed Elution rates of proteins through the column depends on the equilibrium constant of the ionic displacement reactions, and if they are sufficiently different for each protein separation will take place

IE-HPLC has been used successfully for the varietal identification of Australian wheat by Batey (1984) Gliadin elution profiles used for the varietal identification were obtained with a Pharmacia Mono Q column, a wide pore (500Å) anion exchange column Analysis times were reduced to approximately 45 minutes, but the expected good resolution did not materialise, however, characteristic gliadin elution profiles could be obtained for each variety Batey found that the elution profiles obtained were reproducible even after 200 analyses, provided the column was washed regularly with strong solution of counterion

Some work has also been carried out on the use of anion exchange HPLC for barley varietal identification (Wingad *et al* , 1986, Skerritt *et al.*, 1988) but only a few samples were analyzed in each case Wingad reported a small difference between the hordein elution profiles of two European barley varieties and Batey showed that three Australian varieties had characteristic elution profiles It is not clear, however, from the published data whether these profiles are reproducible or if other barley-varieties have also characteristic elution profiles While the results are far from conclusive they nevertheless indicate that the use of IE-HPLC for barley varietal identification is a possibility

1.1.1d Chromatofocusing

Chromatofocusing is a variation of IE-HPLC which separates proteins on the basis of their isoelectric point (pI) rather than net charge The matrix is a weak anion exchanger which generates a linearly descending pH gradient along the column When a protein is applied to the column, at a pH above its pI, it will be absorbed When a descending gradient is started by the addition of a special buffer, the protein will migrate down the column to a point where the pH of the eluent corresponds to the pI of the protein Beyond this point the molecule becomes negatively charged and it cannot migrate further, until its isoelectric point is reached again by the descending pH gradient In this manner protein will elute in a narrow band in order of decreasing isoelectric points Hence, if there is a significant difference between the pI values of extracted hordeins, it should be possible to obtain a well resolved elution profile for each barley variety. While chromatofocusing has been used successfully for the resolution of proteins which could not be separated by other methods, to the best of our knowledge, it has not been used to date for varietal identification

A possible advantage of chromatofocusing over other methods is that the

condition of the column can be determined independently during each run by monitoring the pH of the gradient with an available pH monitor If the slope of the pH gradient changes for any reason the elution profile will no longer be comparable to preceding profiles Another advantage of chromatofocusing is that the final pH of the eluent is below 8, hence polyphenols, which are mainly neutral at this pH, should not be absorbed onto the column

1.1.2 Objectives

It was pointed out in section 1 1 1b that RP-HPLC has been used successfully for the varietal identification of Canadian and European barley varieties However, analysis times of the reported methods are long and there is still a question mark over column stability Analysis times were halved by using IE-HPLC (section 1 1 1c), but characteristic hordein elution profiles were obtained for only two European barley varieties and profile repeatability and column stability were not investigated. Neither RP-HPLC nor IE-HPLC has been used so far for the identification of barley varieties grown in Ireland

The objective of part 1 of the present work is to evaluate if RP-HPLC and/or IE-HPLC hordein elution profiles can be used for the routine identification of Irish barley varieties, and to establish if analysis times and column stabilities can be improved enough to enable us to use the technique for routine barley varietal identification

1.2 MATERIALS AND METHODS

1.2.1 Materials

Barley samples, all certified varieties, were obtained from the breeder or from the Department of Agriculture, Forestry and Food All chemicals - unless otherwise stated were of "Analar" grade

1.2.2 Apparatus

Separations were carried out by using a Mono-Q (50 x 5 mm) anion exchange (50 x 5 mm), Mono-S (50 x 5 mm) cation exchange, Mono-P (200 x 5 mm) chromatofocusing columns (Pharmacia Biosystems Ltd , Milton Keynes, U K) and BioRad Hi-Pore C4 (250 x 5 mm) (BioRad Ltd , Watford U K), and Vydac TPC4 (250 x 4 6 mm) (The Separations Group, Hesperia, CA, U.S A) reverse phase columns Solvent delivery pumps, gradient controller and detector used with ion exchange and

chromatofocusing columns were all part of the Pharmacia FPLC system. Waters M45, 6000A pumps, 441 UV detector, 720 system controller and Shimadzu 6A automatic injector were used with the reverse phase column.

1.2.3 Sample extraction

Hordeins for ion exchange chromatography and chromatofocusing from single seed or barley flour (0 1 g) were extracted with 0 3 and 0 4 ml of equilibrium buffer respectively, for 3 hours or overnight at room temperature Hordeins for reverse phase chromatography were extracted from barley flour or seed with 0 4 ml or 0 3 ml of 55% isopropanol - containing 1% DTT (dithiothreitol) at 60°C for 30 minutes, with vortexing at 10 minute intervals In each case the samples were centrifuged at 20,000 r p m for 5 minutes, and for ion exchange chromatography they were filtered through a 22 mm membrane, using Pharmacia membrane filter units

1.2.4 Chromatographic conditions

1.2.4a Ion exchange - HPLC

For anion exchange chromatography the equilibrium buffer was 0 01 M CAP (3-(cyclohexyl amino)-propane sulphonic acid), containing 1 M urea adjusted to pH 10 4 with 1 M and the eluting buffer was 0 01 M CAP containing 1 M urea and either 0 5 M sodium chloride, or 0 5 M sodium acetate, or 0 5 M sodium bromide Hordeins were eluted with linear gradient of 0 to 25% eluting buffer in 22 minutes The column was washed after each elution with a linear gradient of 25 to 100% eluting buffer in 8 minutes followed by injections of 3 x 0 5 ml 2 M sodium chloride The flow rate was 1 ml/min and typical injection volume 50 µl

For cation exchange chromatography the equilibrium buffer was 0 05 M acetic acid, or 0 05 M lactic acid, or 0 5 M malonic acid, adjusted to pH 5 0, 4 0 and 2 5, respectively with 1 M hydrochloric acid The eluting buffer was the same as the equilibrium buffer in each case with 1 M sodium chloride as counterion Hordeins were eluted with a linear gradient of 0 to 25% eluting buffer in 22 minutes The column washing procedure, flow rate and injection volume was the same as for anion exchange chromatography

1.2.4b Chromatofocusing

Equilibrium and eluting buffers used with the chromatofocusing column for the

three pH ranges were as follows pH 7-5, 0 025 M bis Tris (tris - 2 amino - 2 - hydroxymethyl propane - 1,3 - diol) and Polybuffer 74, pH 4 0, pH 9-6, 0 025 M diethanolamine and Polybuffer 96, pH 6 0, pH 9-8, 0 025 M diethanolamine and Pharmalyte 8, Polybuffer 96, pH 8 0 Eluent buffers were made up by diluting 10 mls concentrated Polybuffer 96 or Polybuffer 74 or 1 ml Pharmalyte 8 and 5 2 Polybuffer 96 to 95 ml with distilled water, adjusting the pH to the specified value with 2 M hydrochloric acid and adding more distilled water to make a total volume of 100 ml Equilibrium buffers were adjusted to 0 1 pH unit higher than the starting pH (i e 9 1 or 7 1) with 2 M hydrochloric acid Pre-gradient and elution eluent buffer volumes were 9 and 25 ml, 3 and 25 ml, 3 and 23 ml for the pH ranges of 9-6, 9-8 and 7-5, respectively

Before the injection of hordeins column pH was stabilised at the starting pH (i e 9 or 7) with the equilibrium buffer, the flow of equilibrium buffer was stopped, and the specified volume of pre-gradient eluent buffer was passed through the column Hordeins were then injected and eluted with the volume of eluent buffer specified for the particular pH range After all the eluent was added the column was washed with the equilibrium buffer until the pH stabilised at the starting pH Typical injection volumes were 50 μl and flow rate was 0 5 ml/min Pharmalyte and Polybuffer are trade names of Pharmacia Biosystems *Ltd*

1.2.4c Reverse phase - HPLC

Equilibrium and elution solvents were 15% and 80% (v/v) aqueous acetonitrile with 0 1% trifluoroacetic acid each and the optimised linear elution gradients were of 25 to 49% eluent in 45 minutes and 25 to 48% eluent in 30 minutes were used for the BioRad and the Vydac columns, respectively After each run the column was flushed with 5 mls 100% eluent and 5 mins linear gradients were used to increase eluent to 100% and decrease it from 100 to 25% Flow rate was 1 ml/min and injection volume 15 μl in each case

1.2.5 Column cleaning

1.2.5a Anion exchange and chromatofocusing column

Flow was reversed, the column was rinsed with 5 ml distilled water, and flow rate was adjusted to 1 ml/min for ion exchange and 0 5 ml/min for chromatafocusmg columns 1 ml 2 M sodium chloride, 1 ml 2 M sodium hydroxide, 1 ml 2 M sodium chloride and 1 ml 75% (v/v) acetic acid solutions were injected in that order, and the column was washed with 5 ml distilled water between each injection The procedure was repeated until stable baseline was obtained and the resolution of standard proteins was satisfactory

In the case of ion exchange columns, a sawtooth gradient of 100% (v/v) water to 100% isopropanol to 100% water in 40 ml, each with 1% TFA was found to be effective if the above procedure did not restore resolution

1.2.5b Reverse phase columns

The Bio-Rad column was cleaned regularly by washing with 50 ml methanol and during the wash four injections of 2 ml DMSO were made After the last injection the column was washed with an additional 50 ml methanol No cleaning was required for the Vydac column so far, apart from the flushing with approx 5 ml 100 % eluent (80% acetonitrile 0 1 % TFA) after each sample

1.3 RESULTS

1.3.1 Separation of hordeins by RP-HPLC

1.3.1a Hordein profiles with BioRad C4 columns

Hordein elution profiles of twenty two different barley varieties were determined by RP-HPLC with a C4 BioRad column (25 x 0 46 cm) The list included the varieties grown here at present, others that were grown here or in Britain in the past, and some promising varieties which had not yet been commercialised A long column (25 cm) was used because previous work on the identification of European barley varieties suggested the use of a column of the same length (Allison and Bain, 1986) The C4 bonded phase matrix was recommended for the separation of proteins in the molecular weight range of hordeins (Vydac, 1991)

Initially hordein extraction procedures and elution conditions developed for C18

columns were used (Allison and Bain, 1986) but it was found that elution gradients, steeper than those reported improved resolution and shortened retention times Each of the twenty two varieties examined here showed a characteristic elution profile, albeit the differences between some are very small Profiles of the varieties grown here in the past two years along with some other varieties are shown in Figure 1a and 1b The differences between hordein elution profiles of Magda and Blenheim, the resolved shoulder of peak no 3 and the relative size and shoulder of peak no 5 are very small (Figure 2) However even these small differences were consistently reproducible Thus it should be possible to use RP-HPLC elution profiles to distinguish between varieties even with minute differences in hordein composition

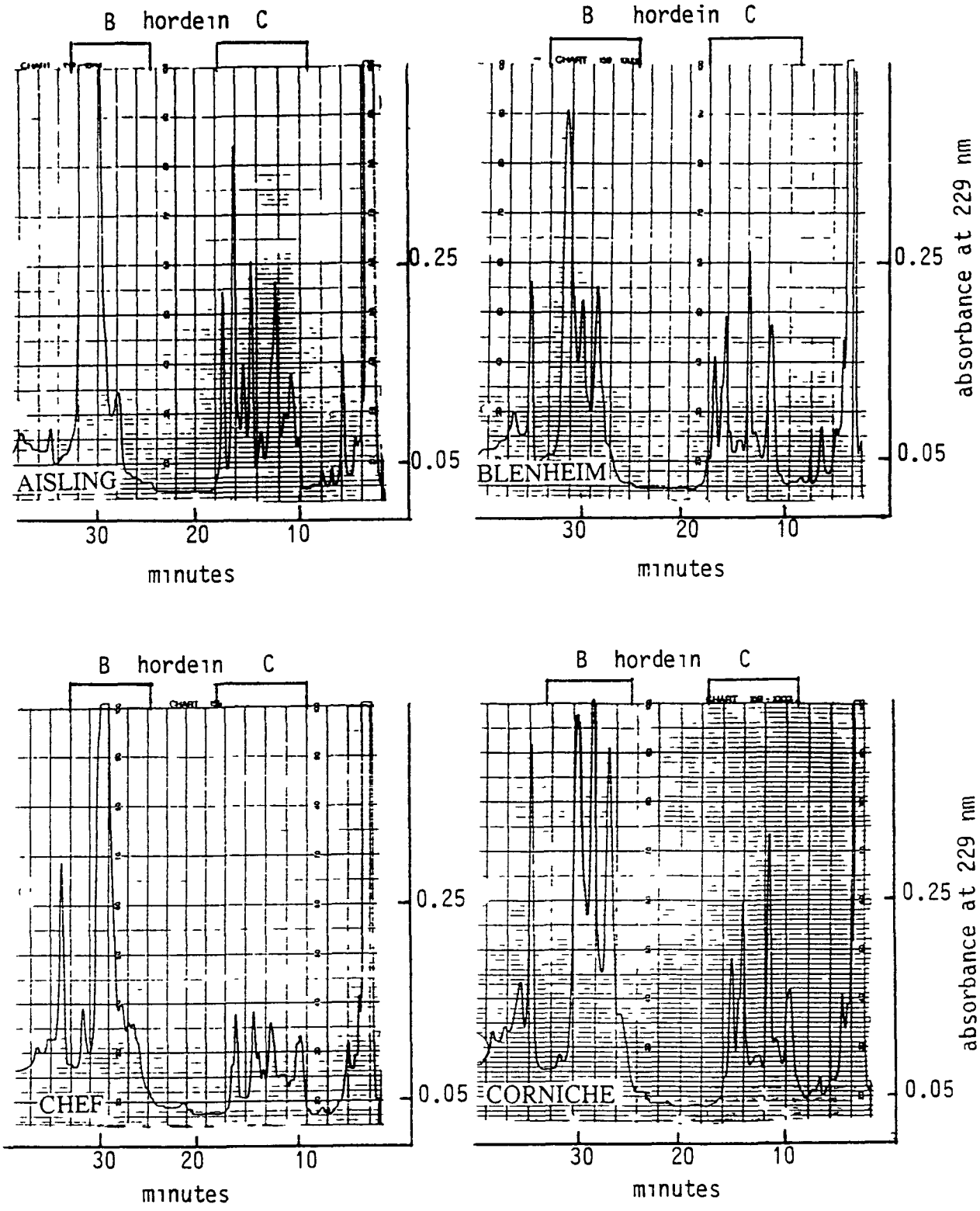
The obtained hordein elution profiles, with two groups of peaks have the same general characteristics as those reported for Canadian (Marchylo 1984) and British (Allison and Bain 1986) barley varieties The two principal groups of peaks have been isolated and analyzed by electrophoresis (Marchylo and Kruger, 1984) and they were found to correspond to the C (70,000-90,000 daltons) and B (30,000-70,000 daltons) sets of bands of the electrophoretic pattern There is a third group of peaks between C and the solvent front (Figure 1) which are equivalent to the A set of electrophoretic bands These peaks, however, change with each extraction and are of little analytical value

While the BioRad 304 reverse phase column gave very good resolution and faster retention times than hitherto reported, it was not without disadvantages Regular cleaning with DMSO was required to retain reproducible elution profiles and after about 150 analyses the groups of peaks could not be resolved any longer, even after extensive cleaning Furthermore, the column which costs about £500 is very expensive and frequent replacements would make varietal identification by RP-HPLC very costly It was necessary, therefore, to examine other columns

1.3.1b Hordein profiles with the Vydac C4 columns

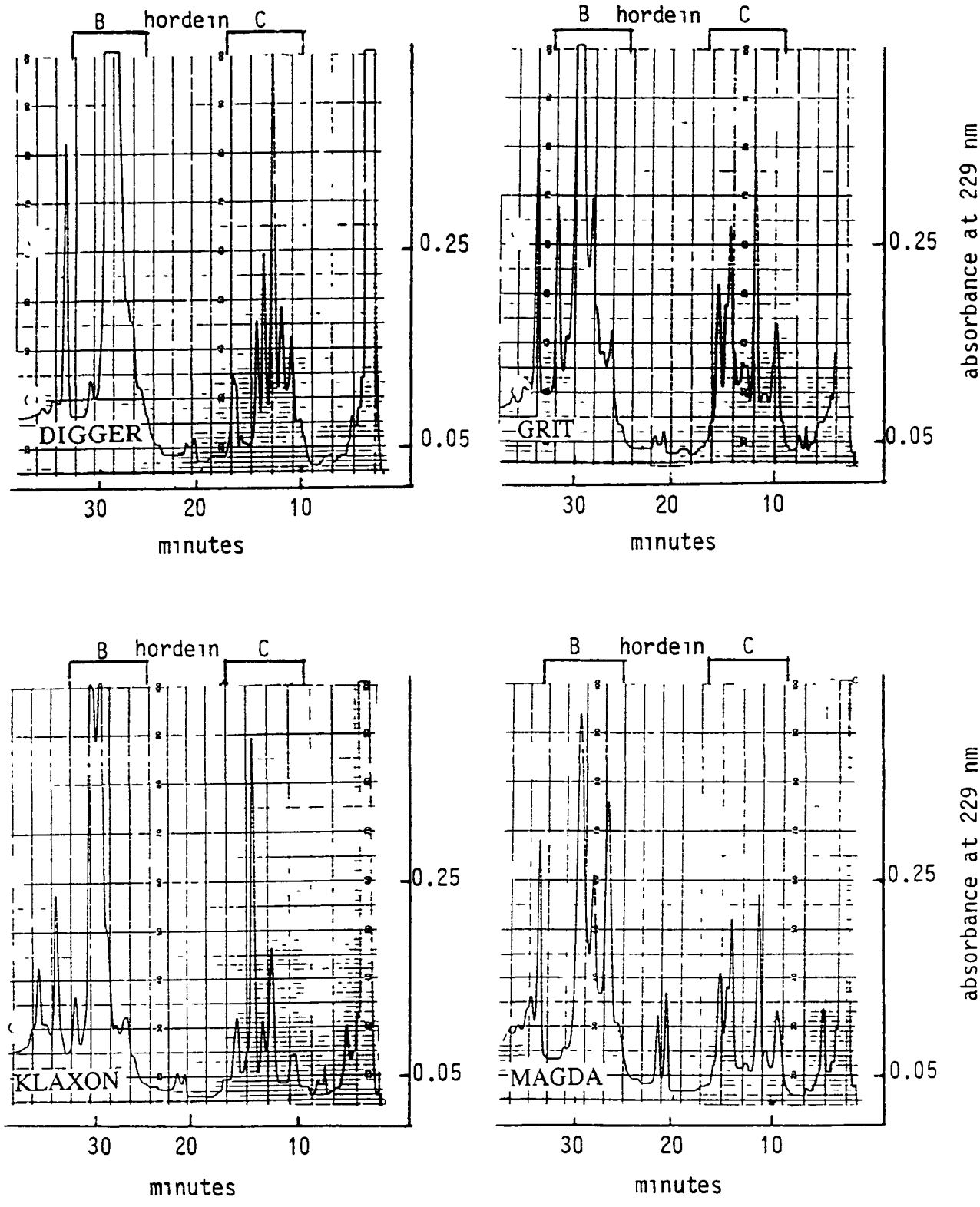
Since the elution profiles obtained with C4, 5μ matrix were very well resolved, a column with the same matrix and dimension but from a different manufacturer was tried out The Vydac 214TP54 column was recommended to use by a user (Gatward, 1992) as a very stable column, and at a cost of £220 is much cheaper than the BioRad column

Figure 1a. RP-HPLC hordein elution profiles of Irish barley varieties



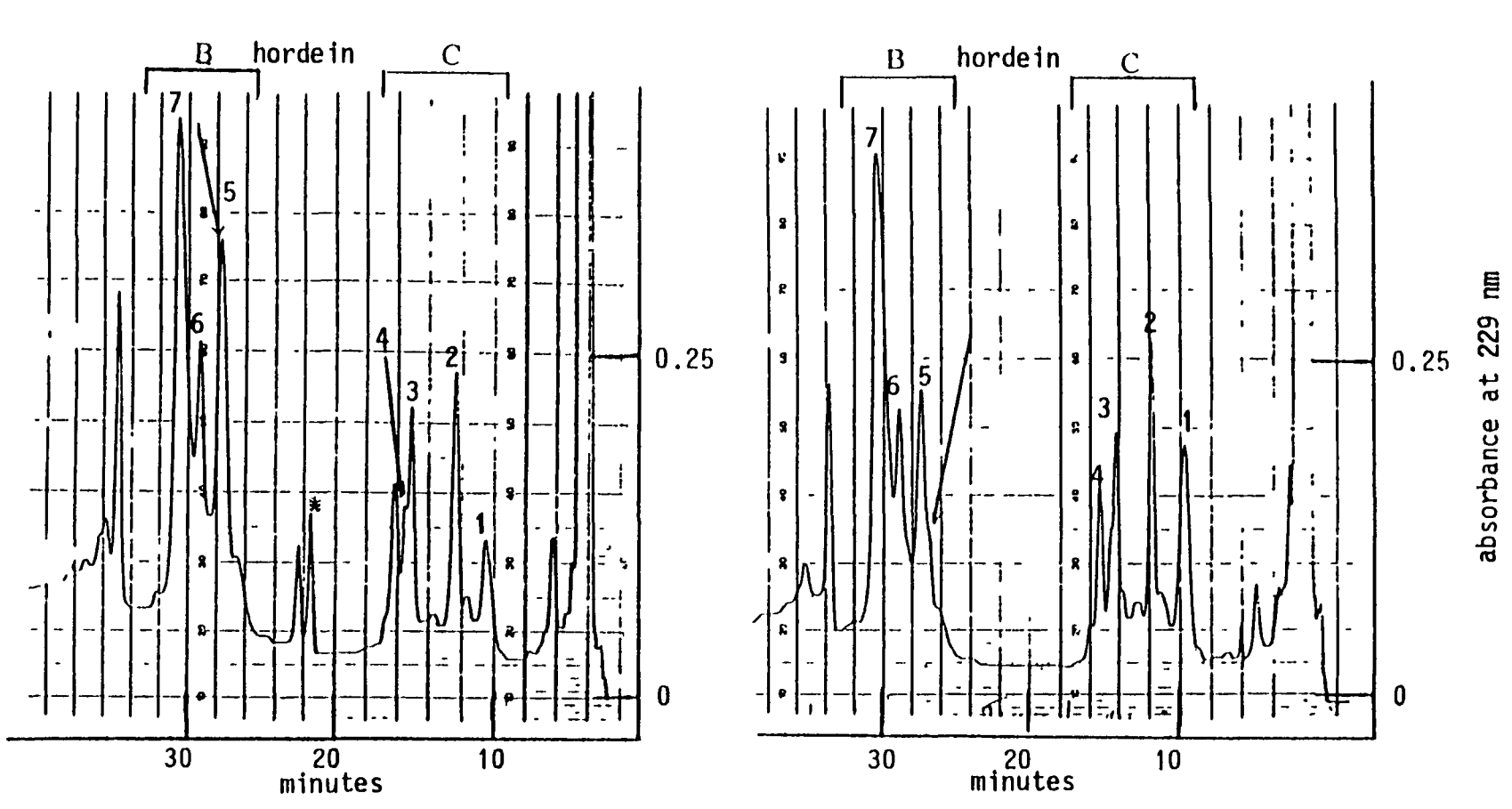
Bio-Rad 304, 250 x 4.6 mm, C4, 300A, reverse phase column, equilibrium solvent, 15% acetonitrile, 0.1% TFA, eluent, 80% acetonitrile, 0.1% TFA, linear gradient, 25-49% eluent in 45 min, flow 1 ml/min.

Figure 1b. RP-HPLC hordein elution profiles of Irish barley varieties



Bio-Rad 304, 250 x 4.6 mm, C4, 300A, reverse phase column, equilibrium solvent, 15% acetonitrile, 0.1% TFA; eluent, 80% acetonitrile, 0.1% TFA; linear gradient, 25-49% eluent in 45 min; flow 1 ml/min.

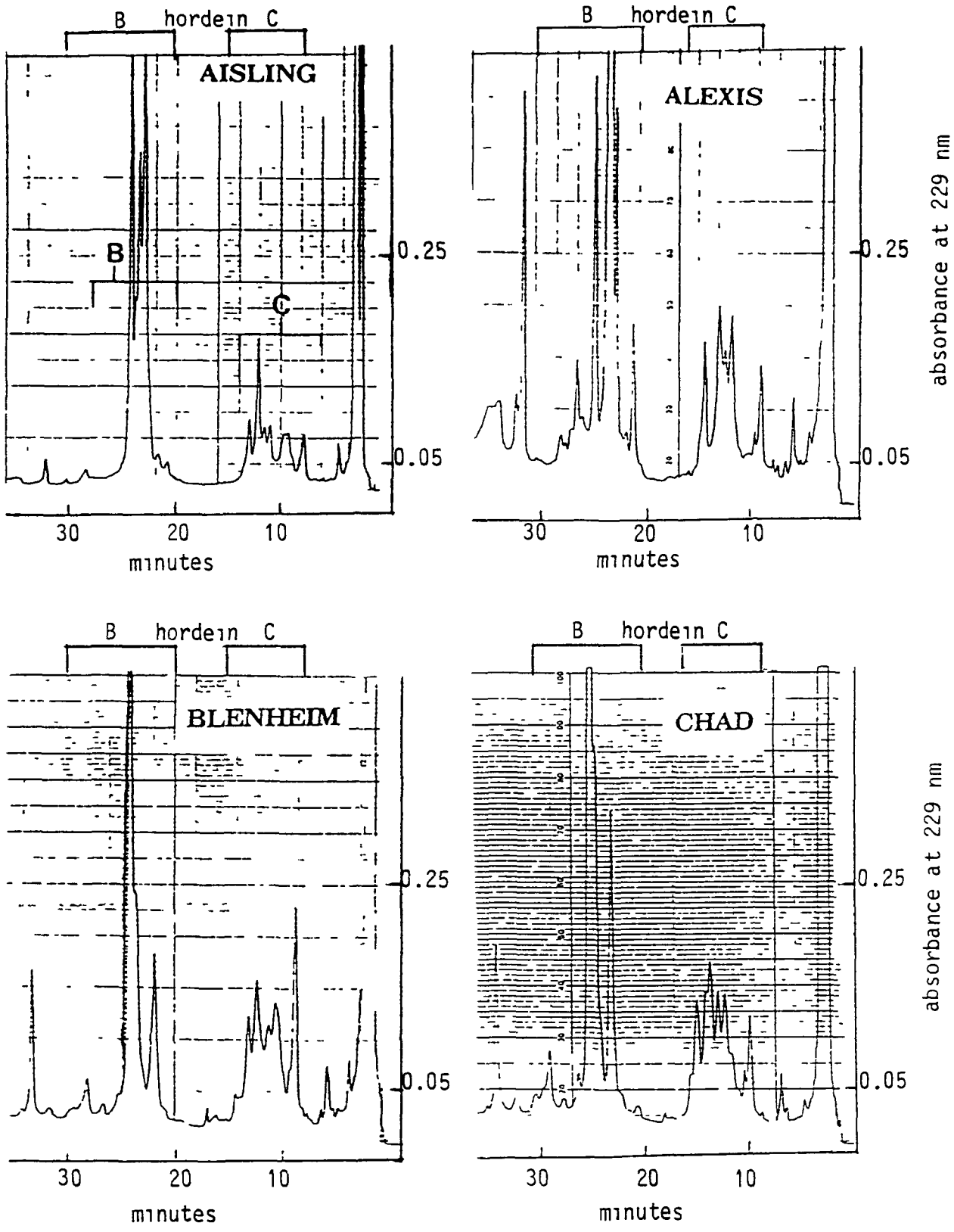
Figure 2. Barley varieties with similar hordein elution profiles (small differences marked with arrows)



Chromatographic conditions listed in Figure 1 were used

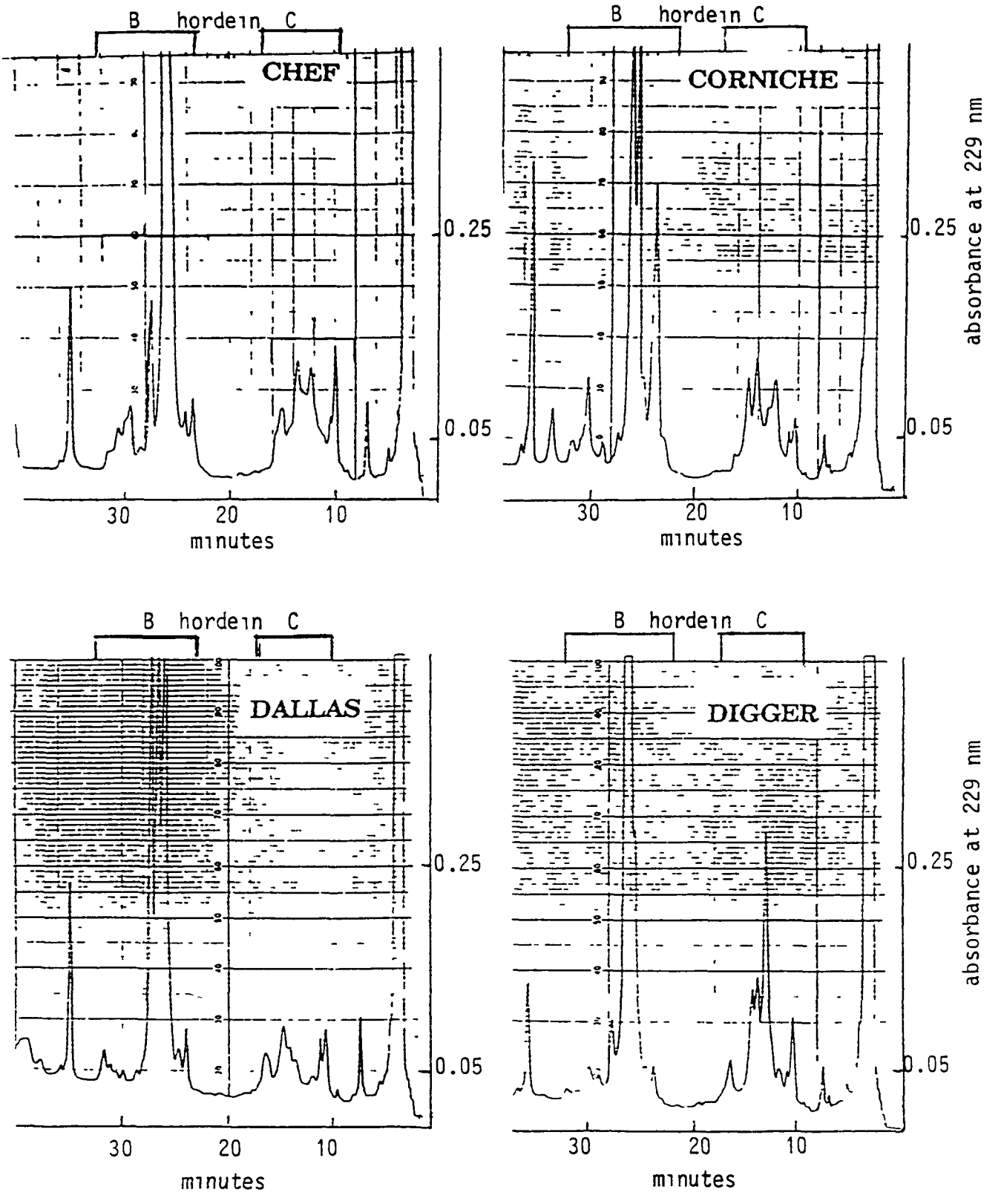
* Not a hordein peak, but probably impurity in mobile phase or extractant. It gradually disappeared.

Figure 3a. RP-HPLC hordein elution profiles of Irish barley varieties



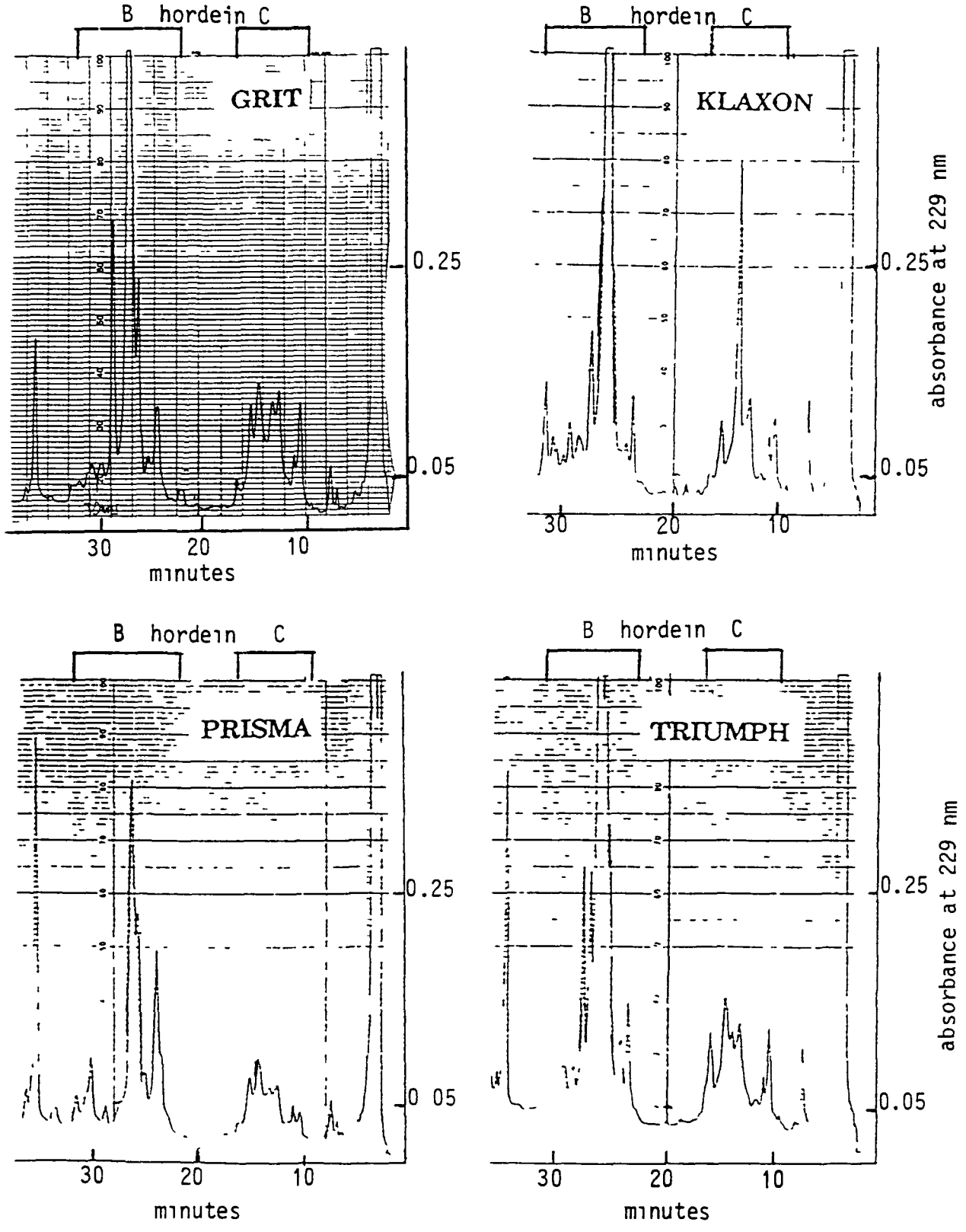
Vydac 214, 250 x 4.6 mm, C4, 300A reverse phase column; equilibrium solvent, 15% acetonitrile, 0.1% TFA; eluent, 80% acetonitrile, 0.1% TFA; linear gradient 25-48% eluent in 30 min; flow, 1 ml/min.

Figure 3b. RP-HPLC hordein elution profiles of Irish barley varieties



Vydac 214, 250 x 4.6 mm, C4, 300A reverse phase column; equilibrium solvent; 15% acetonitrile, 0.1% TFA, eluent, 80% acetonitrile, 0.1% TFA; linear gradient 25-48% eluent in 30 min; flow 1 ml/min.

Figure 3c. RP-HPLC hordein elution profiles of Irish barley varieties



Vydac 214, 250 x 4.6 mm, C4, 300A reverse phase column; equilibrium solvent; 15% acetonitrile, 0.1% TFA; eluent, 80% acetonitrile, 0.1% TFA; linear gradient 25-48% eluent in 30 min; flow 1 ml/min.

Hordein elution profiles of barley varieties grown in Ireland at present and of some older varieties obtained with the Vydac column are shown in Figure 3. Retention times are somewhat shorter than those obtained with the BioRad column, mainly because the slope of the elution gradient had to be increased to prevent excessive broadening of hordein C peaks The resolution of the groups of hordeins, especially C hordeins is considerably poorer with the Vydac column than with the BioRad (Figure 4), but the differences between the elution profiles of different varieties are still very obvious Even small difference such as between Chad and Blenheim (Figure 3) are still very much in evidence, in spite of the poorer resolution

While the Vydac column would not be an obvious choice if hordein resolution alone was the objective, we found it to be a far more stable column than the BioRad. It was not necessary to carry out regular cleaning with DMSO, and even after 200 runs the resolution did not deteriorate and hordein profiles were nearly identical to those obtained with the new column (Figure 5) Therefore, it is considered that the Vydac column would be more suitable than the BioRad column for routine varietal identification of barley samples, for poorer resolution - as long as elution profiles are clearly different - is not as much of a disadvantage as poor column stability

1.3.2 Rapid ion exchange separation of hordeins

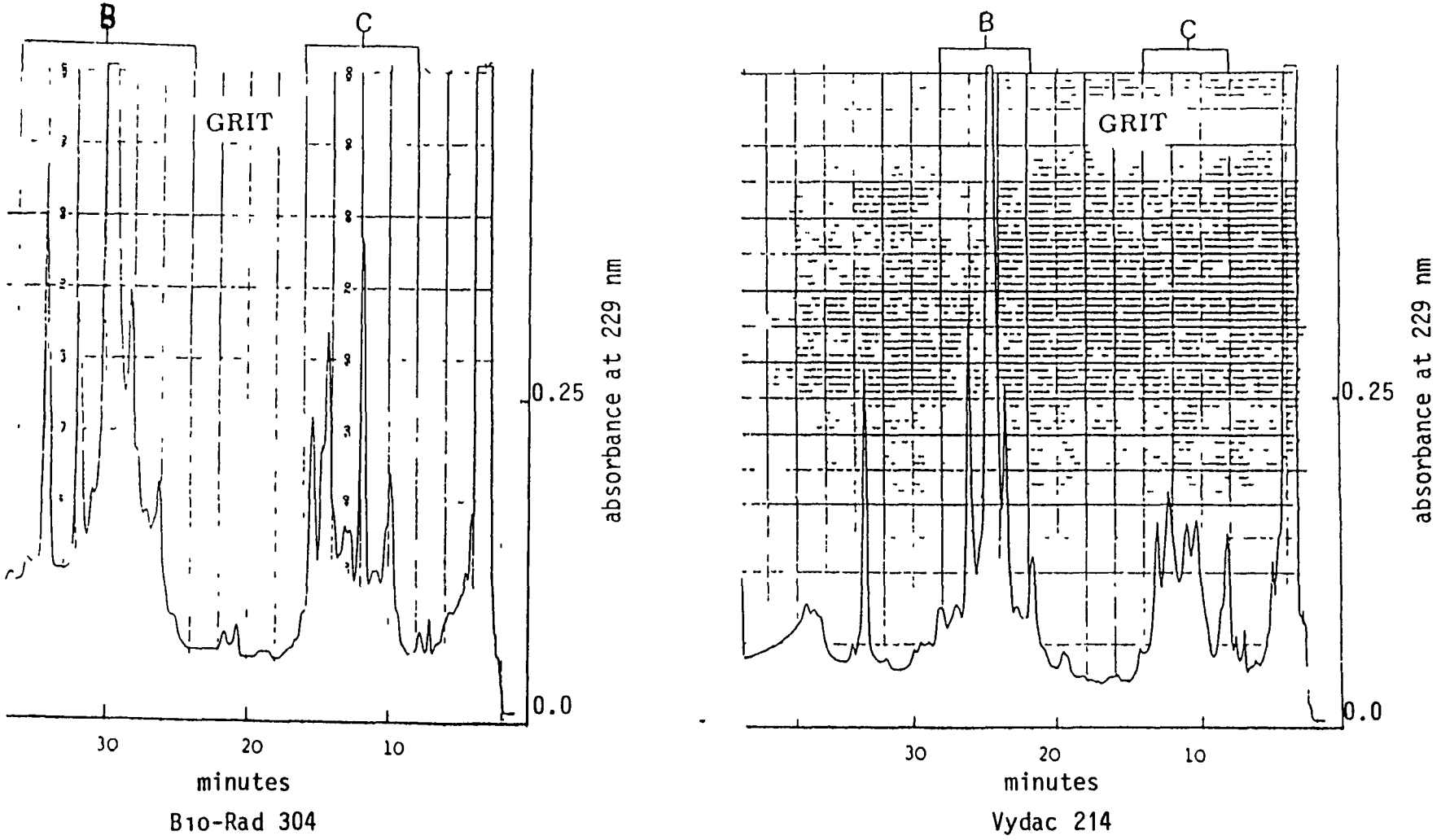
1.3.2a Hordein profiles with wide pore anion exchange column

Before barley hordein elution profiles could be determined the optimum pH for the resolution of extracted hordeins needed to be established Elutions of hordeins from the barley variety Grit were carried out at pH's 7 0, 8 0, 9 0, 9 5 and 10 4 using buffers and conditions reported by Batey (1984) Resolution improved with increasing pH and the optimum pH was found to be 10 4

Poor resolution at lower pH values is probably due to a high proportion of arginine residues (pK =12 5) which remain mainly positively charged up to relatively high pH (Batey, 1984) Positively charged sites are repelled by the charged groups on the anion exchange resins and the respective proteins elute much faster without resolution

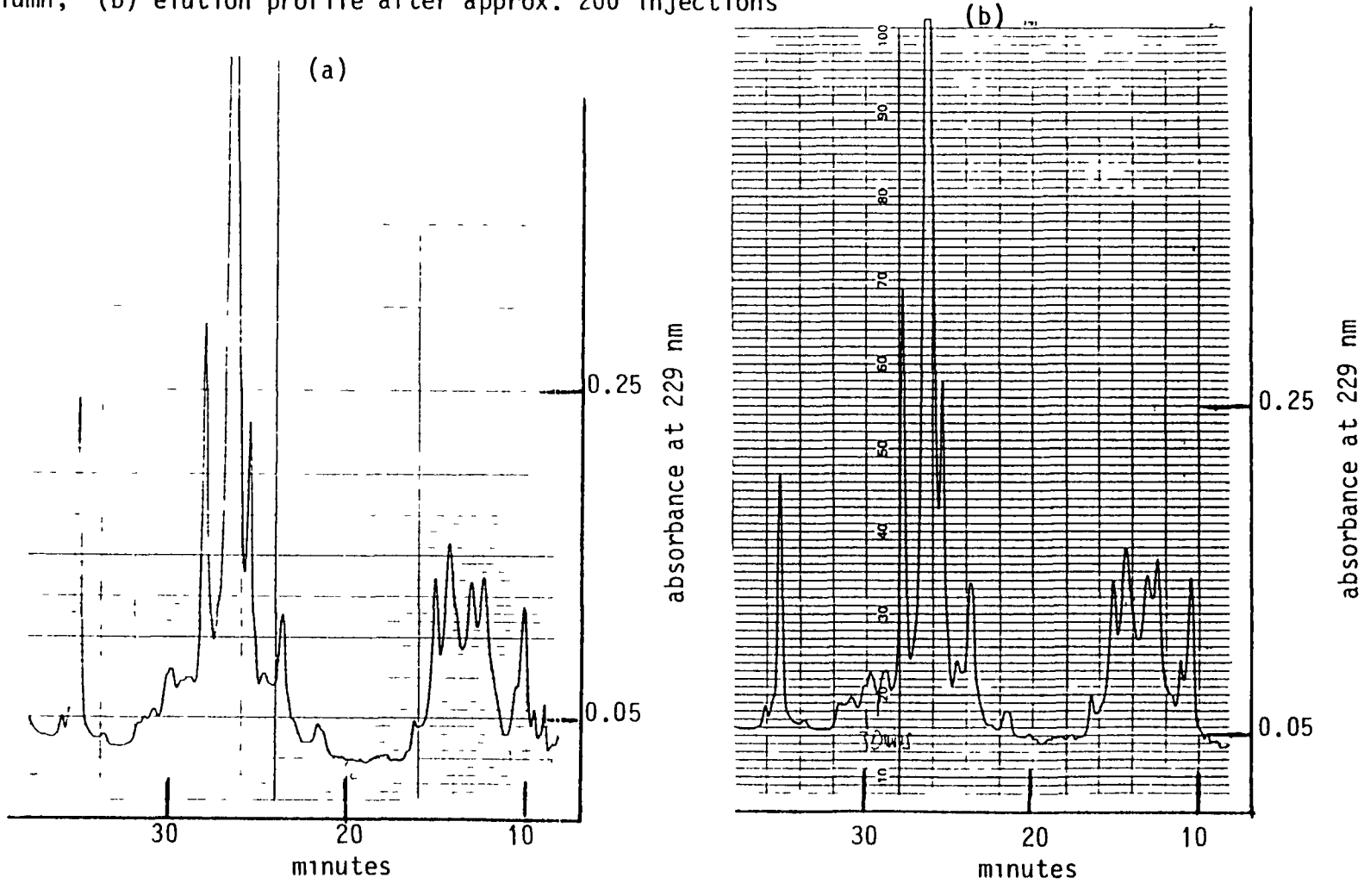
Hordein elution profiles of two different barley varieties, Magda and Grit, obtained using the optimised conditions are shown in Figure 6 It is evident from the chromatograms that the two varieties can be easily identified from their anion

Figure 4. Hordein elution profiles of barley variety Grit obtained with Vydac and Bio-Rad wide pore columns



The two columns had the same dimensions (250 x 4.6 mm) and were both packed with C4, 300A reverse phase material. Chromatographic conditions listed in Figures 1 and 3.

Figure 5. Repeatability of hordein profiles, barley variety "Grit"; (a) hordein elution profile obtained with new column; (b) elution profile after approx. 200 injections



Chromatographic conditions listed in Figure 3 were used

exchange hordein profiles provided these are reproducible The chromatograms are better resolved than those obtained by Wingad (1986) which is probably due to the larger pore size of the Mono Q resin (500Å) used here than that of the DEAE resin (150Å) used in the previous work It was noted before that reverse phase matrix with average pore size of 100Å was unsuitable for the separation of hordeins (Marchylo *et al* , 1988)

The hordem elution profiles of Magda and Grit (Figure 6) are significantly different, and if reproducible, they could be used for varietal identification However, the resolution deteriorated with each run and after about ten analyses the elution profiles no longer appeared to be different Unfortunately pre-columns are not available with the wide pore anion exchange columns and a thorough column cleaning needed to be carried out each time the resolution deteriorated Recommended column cleaning with strong acids and bases, and proteolytic enzymes caused no improvements The only cleaning procedure we found to be effective was a repeated use of a 1 % (w/v) trifluoracetic acid water-propanol sawtooth gradient (0%-100%-0% w/v) thus indicating that the contaminant was probably soluble in acidified organic solvents

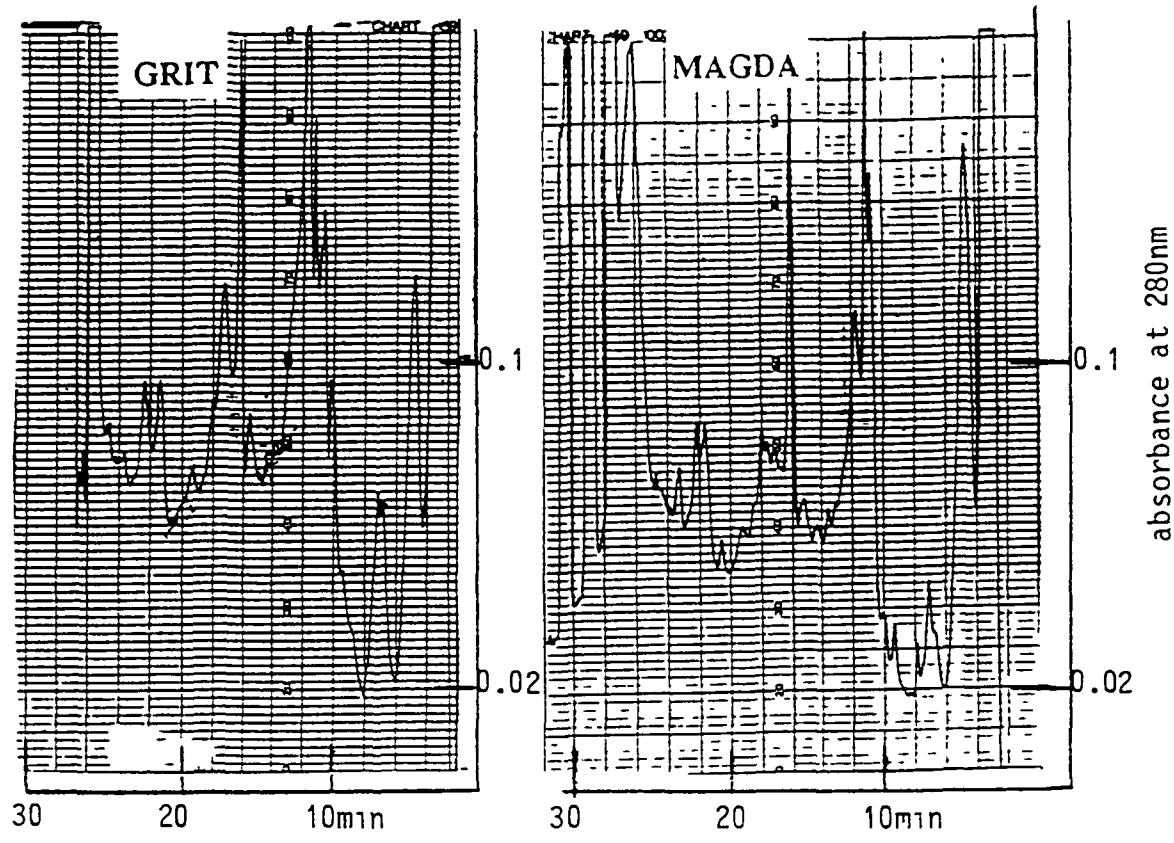
After column resolution was restored with the sawtooth gradient, - different hordem elution profiles could be obtained again for the two barley varieties (Figure 6) However, it is obvious from Figure 6 that the new chromatograms were not identical to those obtained before, and hence, cleaning must have changed the column environment, or the contaminants were not completely removed

After repeated runs column resolution deteriorated again but this time it could not be restored by the extensive cleaning procedure It was necessary to remove the thin brown layer which accumulated on the top of the column, and re-suspend the top 2-3 mm resin before the column could be used again At this point it became obvious that a method has to be found to eliminate, or at least, minimise column contamination before we could proceed further

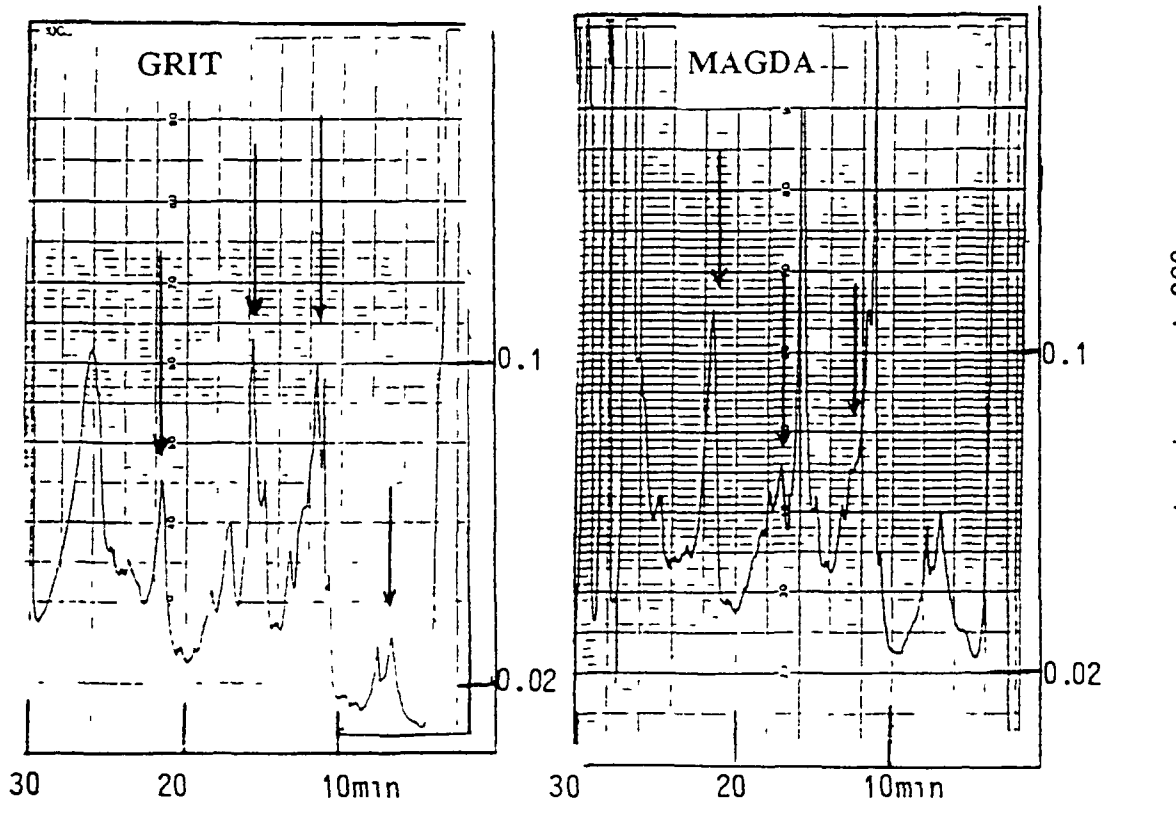
1.3.2b Experiments to minimise column contamination

It was possible to show the effect of repeated runs of barley extracts on the anion exchange column not only by deteriorating hordem elution profiles but also by chromatograms of protein standards Three proteins, transferrin, ovalbumin and β-

Figure 6. Hordein elution profiles of barley varieties by anion exchange HPLC, Mono Q (5 x 50 mm) column



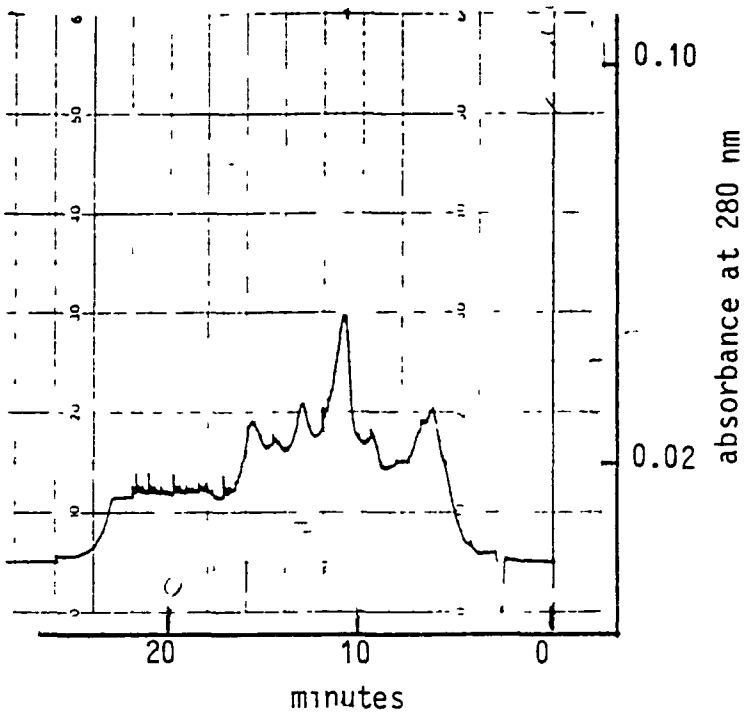
hordein profiles after first column cleaning



hordein profiles after second column cleaning

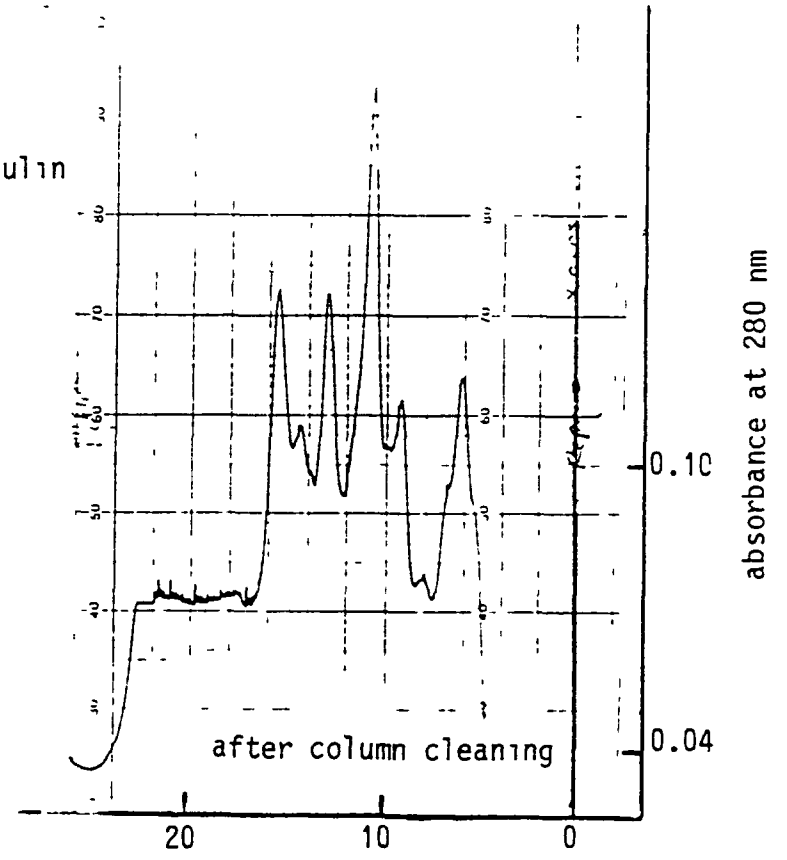
Differences between profiles due to column cleaning are indicated by arrows. Chromatographic conditions listed in section 1.2.4a.

Figure 7. Resolution of test proteins with the Mono Q anion exchange column



before column cleaning (after six injections of barley extract)

100 uls of
1. Transferrin
2. Ovoalbumin
3. B-lactoglobulin
1 mg/ml each



equilibrium buffer; 0.2 m piperazin pH 6.0; elution buffer, 0.2 m piperazin, pH 6 with 0.5 M NaCl; gradient 0.5 - 100% elution buffer in 20 min; flow 1 ml/min.

lactoglobulin were well resolved (Figure 7) just after column cleaning However, after repeated injections of barley mobile phase extracts the peaks corresponding to the three standard proteins became much smaller, broader and very poorly resolved (Figure 7) Removal of the thin brown layer from the top of the column followed by chemical cleaning restored column efficiency and the chromatogram of the standard sample became identical to that obtained before removing the extracts Repeated standard runs did not lead to poorer chromatograms, thus indicating that the observed loss in resolution must be caused by the absorption of materials co-extracted from barley along with hordeins

Attempts were made to prolong column performance by reducing the amount of co-extracted material in the extract by the following methods (i) Extraction times were reduced from overnight to 3 hours, (ii) the extract was diluted by a factor of four with mobile phase, (iii) the 6 %(w/v) urea which probably contributes to an increase of co-extracted materials, was omitted from the extractant While each of these methods reduced the amount of impurities injected into the column, the number of peaks and their heights were also reduced Shorter extraction times or urea free extracts resulted in fewer and smaller peaks and differences between elution profiles of different varieties were not obvious The same results were obtained with diluted extracts

Attempts were also made to examine the effect of aqueous isopropanol hordein extraction on the column The tertiary structure of hordeins must have changed irreversibly in isopropanol, because the resulting chromatogram consisted of a single broad peak Trial elutions with denatured hordeins were equally unsuccessful, because the thiol group of the reducing agent, mercapoethanol, is ionised at pH 10 5 (pKa = 8 5) and the thiolate anion elutes at the same time as hordeins On account of the strong absorbance of the thiolate anion at 280nm the ionised 1% mercaptoethanol completely masks whatever hordein profile might be there UV absorbance of organic sulphides has been reported (Fennel and McCormack, 1949)

Elution of contaminants by using stronger or more "organic" counterions such as bromide or acetate instead of chloride was also considered It was hoped that bromide would dislodge protein contaminants more effectively than chloride Furthermore organic contaminants such as polyphenols would be more soluble in a mobile phase with acetate instead of inorganic counterions, hence less likely to contaminate the column Neither counterion was successful Elution profiles using

bromide as counterions were poorly resolved (Figure 8) and the well resolved elution profile obtained with acetate (Figure 9) counterions could not be reproduced from run to run.

1.3.2c Studies of possible contaminants

As far as it is possible to tell from the evidence obtained so far, the material responsible for column contamination is lodged in the top 1-2 mm of the column A brown layer appears after repeated injections of barley extracts, and when removed column resolution is restored The lodged material probably prevents the absorbance of hordeins in a thin uniform band required for good resolution

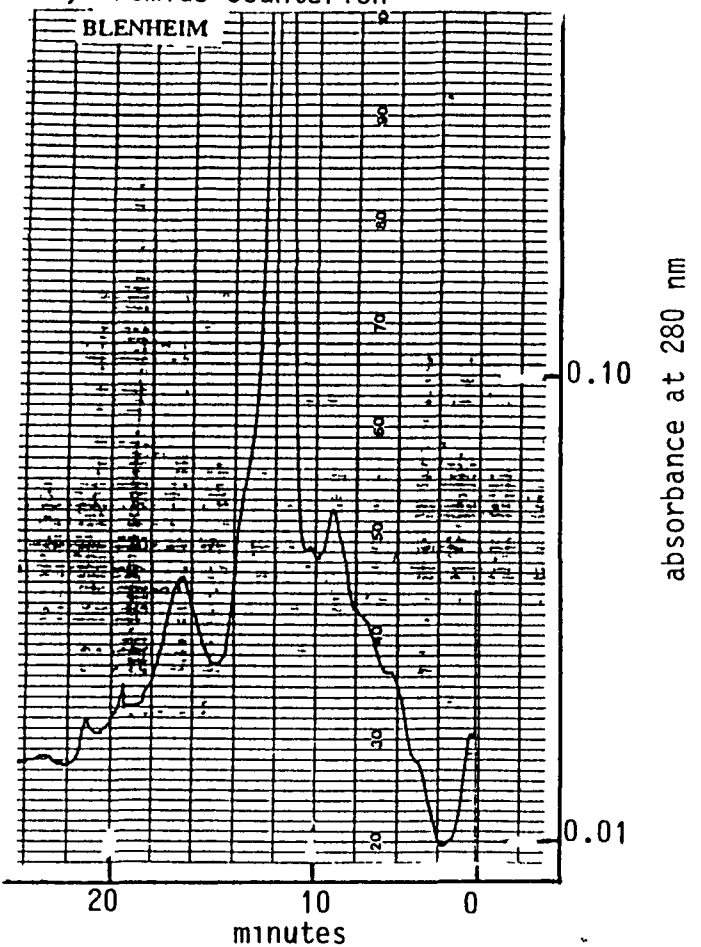
Barley husks and bran contain a wide array of phenolic compounds (Briggs, 1978) and these could be the contaminants responsible for poor resolution Since the pKa's of phenols are between 9 and 10, extracted polyphenols will be in the form of phenolate anions in the mobile phase Polyphenols, however, are large organic molecules, which are not only absorbed by the charged group of the resin but also by the hydrocarbon matrix Hence it might be difficult to dislodge the absorbed polyphenols with the inorganic counterions in the eluent

1.3.3 Hordein profiles with cation exchange columns

Polyphenols have no positive charges in the normal pH range, hence they will not be retained by cation exchange resins Thus, if hordeins could be resolved in the pH range of 2-6, the operational range of cation exchange resins, cation exchange HPLC could be used for barley varietal identification

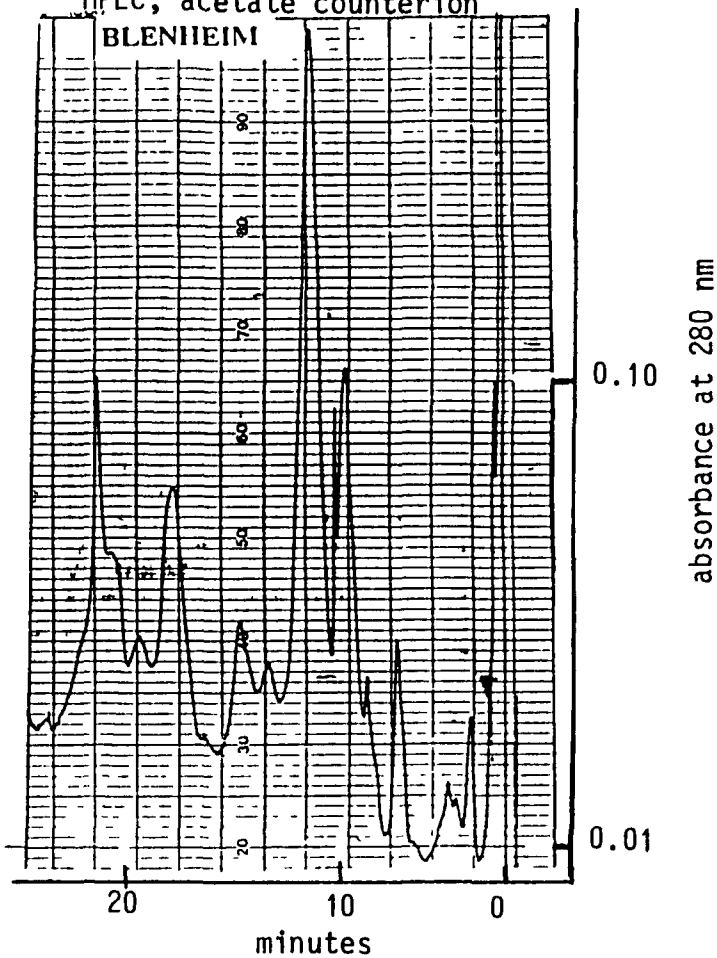
Elutions of hordein extracts from the barley variety "Blenheim" with the starting mobile phase were tried in the pH range 2-6 The Mono S cation exchange column (500Å pore size) was used and the pH range was covered with buffered mobile phases recommended by the manufacturer Only two large frontal peaks were obtained in each case except at pH=5 where the eluted hordeins showed a poorly resolved profile (Figure 10) Although initially some differences were observed between the elution profiles of the three varieties examined, these could not be reproduced consistently On account of the poor repeatability, obtained hordein profiles were of no value for varietal identification and the technique was not investigated further

Figure 8. Hordein elution profile, anion exchange HPLC, bromide counterion



Mono Q (5 x 100 mm) column; equilibrium buffer, 0.01 M CAP, 1 M urea, pH 10.4; eluting buffer, 0.01 M CAP, 1 M urea, 0.5 M sodium bromide, pH 10.4, linear gradient 0-25% eluting buffer in 22 min; flow 1 ml/min.

Figure 9. Hordein elution profile, anion exchange HPLC, acetate counterion



Mono Q (5 x 100 mm) column; equilibrium buffer, 0.01 M CAP, 1 M urea, pH 10.4; eluting buffer, 0.01 M CAP, 1 M urea, 0.5 M sodium acetate, pH 10.4; linear gradient 0-25% eluting buffer in 22 min; flow 1 ml/min.

profiles were of no value for varietal identification and the technique was not investigated further

1.3.4 Separation of hordeins by chromatofocusing

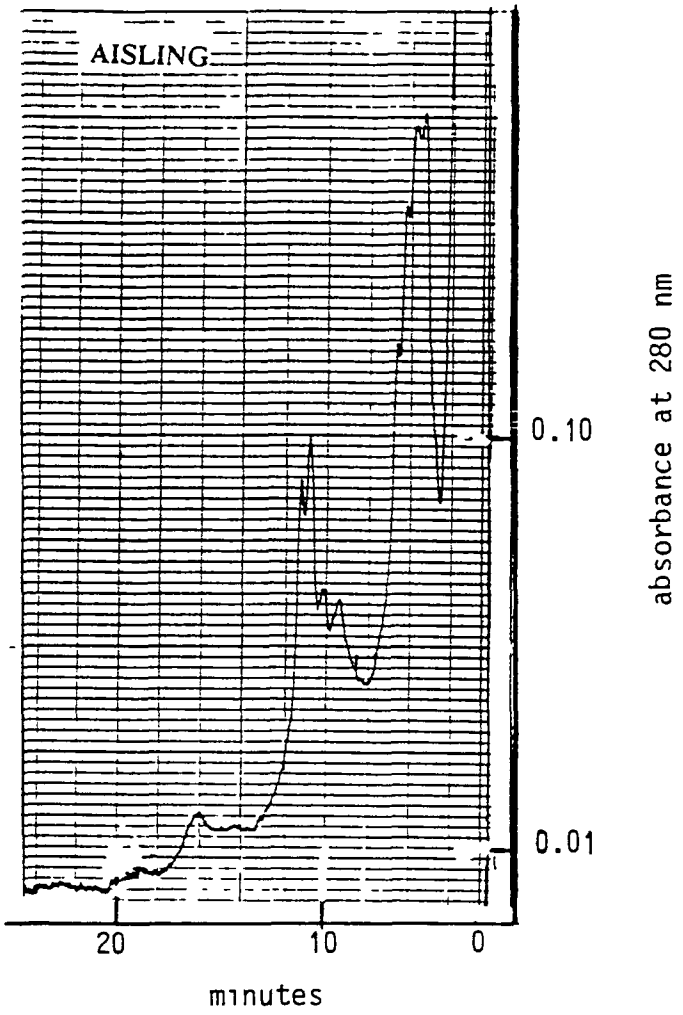
In order to establish the optimum conditions for the resolution of hordeins by chromatofocusing, samples were eluted in the pH ranges of 7-5 and 9-6 In each case hordeins were extracted with the equilibrium buffer Only a single peak was obtained in the pH range of 7-5 (Figure 11), but some hordeins were resolved between 9-6 range - albeit over a very narrow range (Figure 11) Accordingly the pK values of hordeins are very close with differences of less than 0 1 pH units

Chromatofocusing hordein elution profiles were obtained for five barley varieties namely Grit, Aisling, Digger and Chef, in the pH range of 9-6 (Figure 12) Each of the examined varieties had a different elution profile which was repeatable on the same day However, if the column was left overnight in the equilibrium buffer the elution profiles were not the same next day, although still reproducible and different for each variety

After about a dozen injections peak widths increased and the resolution started to deteriorate Extensive column cleaning with strong acids and bases and salt solution, as recommended by the manufacturer restored the resolution However, hordein profiles obtained after column cleaning were different from those obtained before (Figure 13) but they were still reproducible and characteristic of each variety It was found that in order to retain resolution, extensive column cleaning was required after every ten runs, and each time the characteristic elution profiles changed

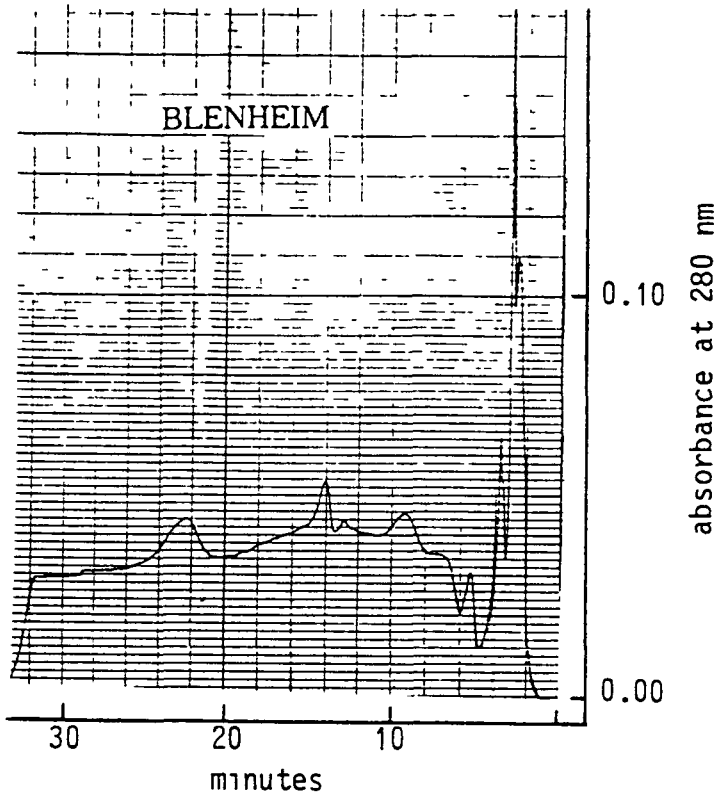
Attempts were also made to expand the elution profile using a narrow range buffer from pH 9-8 While the hordein elution profiles expanded the difference between varieties was minimal and the profile could not be used for varietal identification We have no plausible explanation for the foregoing observation except that the chromatofocusing column used was relatively old and it might no longer be operational in a narrow pH range

Figure 10. Hordein elution profile, cation exchange HPLC

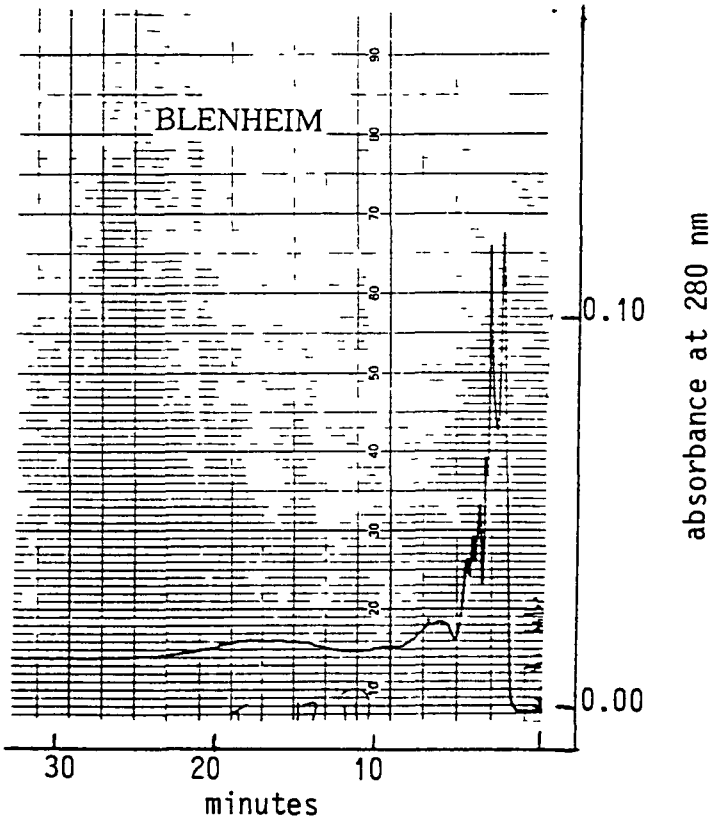


Mono S (5 x 50 mm) column, equilibrium buffer, 0.05 M acetic acid at pH 5; eluting buffer, 0.05 M acetic acid at pH 5, 1 M sodium chloride; linear gradient 0-25% eluting buffer in 22 min; flow 1 ml/min.

Figure 11. Resolution of hordeins by chromatofocusing using Mono P 5 x 200 mm weak anion exchange column

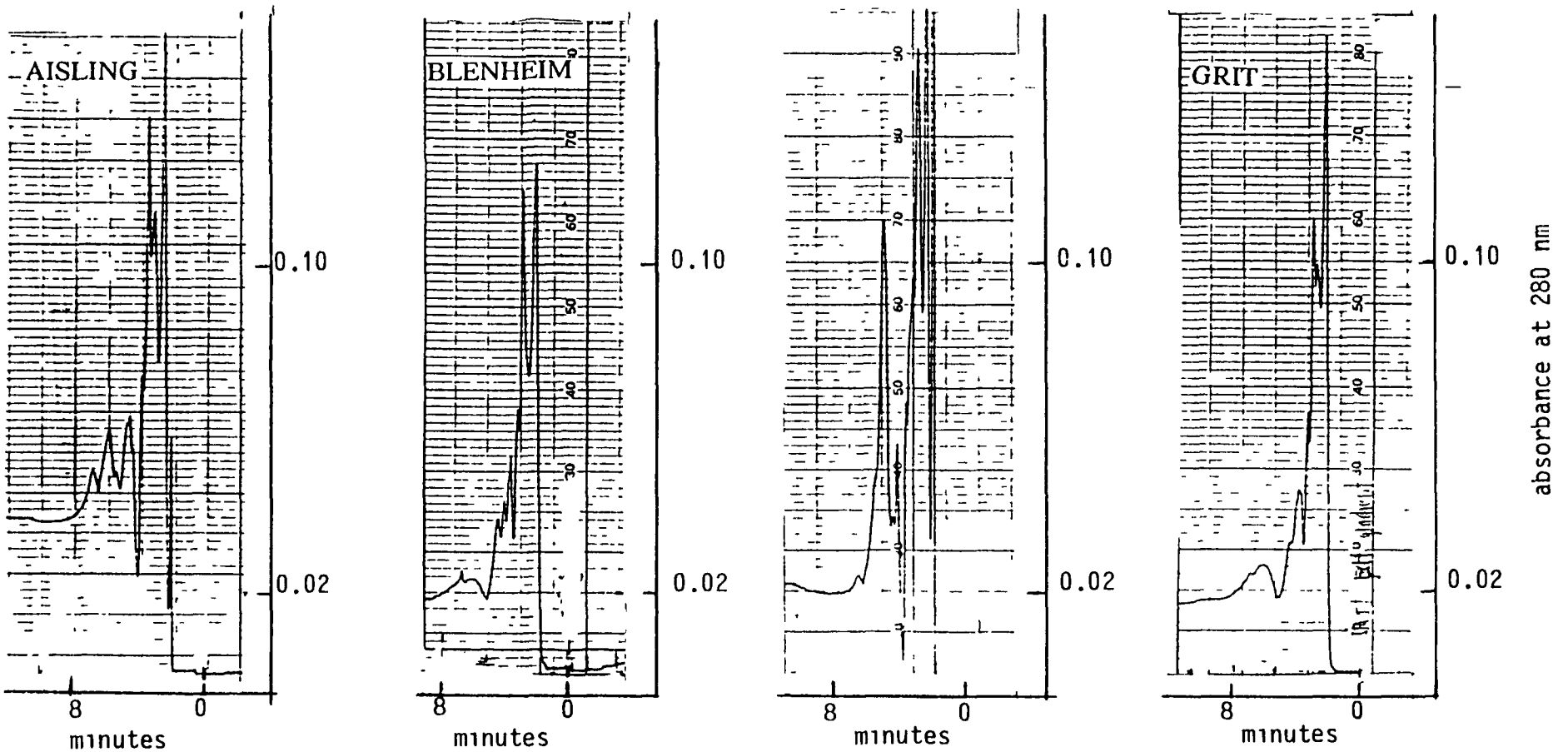


equilibrium buffer; 0.025 M bis Tris pH 7.1, eluting buffer, Polybuffer 74 pH 4; flow 0.5 ml/min + standard lettering



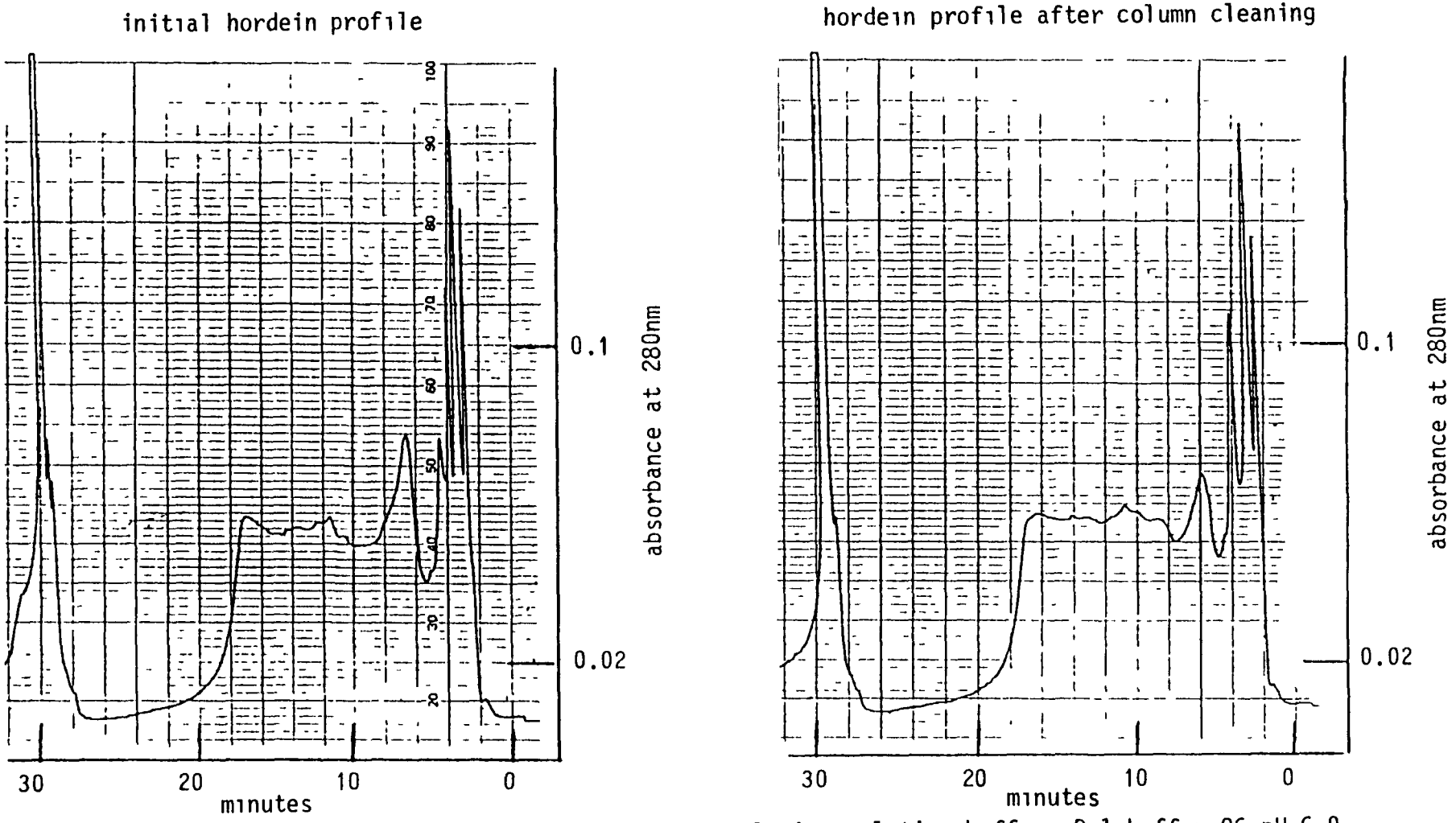
equilibrium buffer; 0.025 M diethanolamine pH 9.5; eluting buffer, Polybuffer 96 pH 6; flow 0.5 ml/min

Figure 12. Hordein elution profiles of Irish barley varieties obtained by chromatofocusing, pH range 9.6



column, Mono P 5 x 200 mm; equilibrium buffer, 0.025 M diethanolamine pH 9.5; eluting buffer, Polybuffer 96, pH 6.0; flow 0.5 ml/min.

Figure 13. Effect of column cleaning on chromatofocusing hordein elution profiles, barley variety Chad, pH range 9-6



column, Mono P (5 x 200 mm); equilibrium buffer, 0.025 M diethanolamine; eluting buffer, Polybuffer 96 pH 6.0, flow 0.5 ml/min.

1.4 DISCUSSION

1.4.1 The use of RP-HPLC for barley varietal identification

1.4.1a Comparison of Vydac and BioRad C4 columns

RP-HPLC using Vydac or BioRad C4 columns and the optimised conditions described in the experimental section was found to be suitable for the separation of hordeins extracted from Irish barley varieties Hordein profiles of the examined barley varieties showed two well resolved groups of peaks with both columns referred to as C and B hordeins, (Marchylo and Kruger, 1985) and the peak patterns corresponding to these two groups were different for each variety (Figures 1a,b and 2a,b,c) Since the peak patterns of the C and B hordeins were repeatable, as long as the column did not show signs of deterioration the obtained hordein elution profiles could be used for varietal identification The peak patterns were sufficiently different for each variety to allow varietal identification by simple visual examination Comparative analysis of the elution profiles, using percent peak areas, and peak area ratios was not necessary

Elution profiles obtained with the BioRad column were considerably better resolved than those obtained with the Vydac column, especially for the C hordeins For example in the case of the variety Blenheim the same number of peaks are present in both the BioRad and Vydac elution profiles, but peaks corresponding to the C hordeins are far better resolved with the BioRad column (Figure 4) Retention times of the BioRad B hordeins as shown in Figures 1 and 2 are about 6 mins longer than the corresponding times with the Vydac column, (Fig 3), but similar times can be obtained by increasing the gradient slope after elution of the C hordein

It is difficult to explain the difference in hordein resolving power between the two columns from the available data Both columns have the same dimensions (25 x 0 46 cm), and are packed with 5 micron 4 alkylsilane bonded silica gel and give nearly identical resolutions of the test protein mixture composed of RNA-ase, lysozyme, BSA, myglobulin and ovoalbumin (Table 1) The somewhat shorter retention times obtained with the Vydac column can be explained by the steeper elution gradient (0-75% ß in 20 min vs 0-100% ß in 30 min) Available data on the physical, and chemical properties of the packing materials (Table 2) indicate that the Vydac packing could have 30 % higher carbon load than the BioRad columns - which implies higher retentivity High retentivity results in slower desorption of absorbed proteins - hence the peak broadening.

However, it is far from clear why the same peak broadening and increased retentivity is not observed with the five test proteins

Table 1: Retention times (min) of selected proteins relative to ribonuclease[1] obtained with C4 RP columns

	Bio-Rad	Δ	Vydac	Δ
Insulin	7 5		7 8	
		2 0		2 3
Lysozyme	9 5		10 1	
		0 8		0 9
BSA	10 3		11 0	
		1 9		2 2
Myoglobin	12 2		13 2	
		2 5		2 9
Ovoalbumin	14 7		16 1	

[1]Data calculated from the manufacturer's column test chromatograms

Table 2: Characteristics of Vydac and Bio-Rad C4 RP columns

	Vydac[1]	Bio-Rad[2]
Carbon loading	3-4%	3%
Pore diameter	300 Å	330 Å
Pore volume	0 5 - 0 6 cm^3/g	0 66 cm^3/g
Particle surface area	80 - 90 m^2/g	90 m^2/g

[1]Data obtained from manufacturer

[2]Data from 1985 Bio-Rad catalogue

While very well resolved hordein elution profiles can be obtained with the Bio-Rad column, poor column stability is a major drawback Hordein resolution tends to deteriorate after about 20-30 analyses and column performance can only be restored by extensive cleaning with DMSO Prolonged use of the column (about 150 analyses) for varietal identification led to complete loss of resolving power, and column performance could not be restored by standard column cleaning methods Other workers (Gatward, 1992) had similar experiences with several reverse phase columns, but the makes of the particular columns were not specified

In contrast to the Bio-Rad column the Vydac column was found to be stable, and even after 200 runs the same elution profiles could be obtained as with the new column. The column did not show gradual loss in resolution, and a short wash cycle after each analysis was sufficient to maintain performance Although the resolution of the C hordeins was not as good as that obtained with the Bio-Rad column, the peak patterns were sufficiently different for each variety to allow easy visual identification

Since the Vydac column is far more stable than the Bio-Rad column and it has the added advantage of much lower cost (£220 vs £500), in our opinion it is more suitable for routine barley varietal identification When a large number of samples need to be analysed, column stability is more important than improved resolution, as long as hordein elution profiles are clearly different However, when varieties with very similar hordein profiles need to be differentiated, it might be necessary to use a column with high hordein resolving power, such as the Bio-Rad column

1.4.1b Influence of reverse phase material on varietal analysis times

Reported analysis times for barley varietal identification, which includes hordein elution, column washing, and equilibration, with C18 column (Marchylo and Kruger, 1984, Allison and Bain, 1986) range from 100 to 110 mins Long analysis times are not practical for the determination of varietal purity, where at least twenty seeds need to be analysed from each sample However, it was possible to reduce analysis time to 50 mins, without noticeable loss in resolution by using a C4 (Vydac or Bio-Rad) instead of C18 columns Analysis times of 45 mins have been also reported with the C18 column (Skerritt *et al*, 1986) These were obtained by using steeper elution gradients, but resolution was poor and the obtained profiles could not be used for varietal identification

Shorter analysis times obtained in the present work with C4 columns compared to those obtained with C18 columns (Allison and Bain, 1986, Marchylo and Kruger, 1984) is mainly due to the steeper gradient that can be used for the separation of hordeins with the former without loss in resolution Gradients, total elution times and elution times for C hordeins with C4 and C18 columns are listed in Table 3 It is valid to use different barley varieties to compare C hordein elution times from different columns, because it was found that C hordeins from all the studied varieties had about the same initial and final elution times either with C4 (Figs 1 and 3) or with C18 columns (Allison and Bain, 1986, Marchylo and Kruger, 1984) Hordein elution

parameters listed in Table 3 indicate that to obtain good resolution, a much lower gradient needs to be used with the C18 than with the C4 column Considering that approximately the same modifier (acetonitrile) concentration is required to start the elution of C hordeins from the three columns, lower gradient will result in a later start of hordein C elution, hence longer total elution times In order to bring hordein elutions more in line with those obtained with the C4 columns the elution gradient elutions needs to increase from 0 19 to 0 46 However the increased gradient with the C18 column leads to very poorly resolved elution profiles which cannot be used for varietal identification (Skerritt *et al*, 1986)

Table 3: Hordein elution times and gradients with C4 and C18 RP columns

Column	Material	Start of elution of C hordeins	% Modifier	End of elution of C hordeins	% Modifier	Elution gradient	Total elution time*
Vydac	C4	10 min	36 3	18 min	40 2	0 50	28 min
Bio-Rad	C4	10 min	34 7	18 min	37 5	0 35	34 min
Synchropak[1]	C18	24 min	35 8	40 min	39 0	0 19	80 min
Synchropak[2]	C18	16 min	48 5	20 min	51 1	0 46	30 min

*Time needed to elute the last peak of B hordeins
[1] Allison and Bain, 1986 [2] Skerrit *et al*, 1986

The large difference between hordein elution times required to produce well resolved hordein profiles C4 and C18 columns can be explained in terms of protein binding It has been established that alcohol-soluble proteins bind more tenaciously to reverse phase materials with longer alkyl chains (Nice *et al*, 1981) Stronger binding implies slower desorption, hence it should take longer to remove bound hordeins from C18 than from C4 materials Therefore, in order to obtain the same resolution with C18 give enough time for each hordein to elute completely before elution of the next hordein begins If a hordein is not fully desorbed before the next hordein starts to elute, the corresponding bands will not be properly separated, and the result is a poorly resolved elution profile Consequently when the gradient slope is more than doubled with the C18 column (Table 3), individual proteins absorbed by the column will not have

sufficient time to desorb fully before desorption of the next hordein begins, hence the poor resolution

1.4.2 Barley varietal identification and anion exchange - HPLC

Hordein elution profiles obtained by anion exchange HPLC in the present work (Figure 6) indicate that the technique in its present form is not suitable for barley varietal identification While characteristic hordein profiles can be obtained for different barley varieties with a new column or immediately after column cleaning (Figure 6), resolution deteriorates rapidly, and after 5-10 runs all hordein elution profiles look alike Elaborate column cleaning restores resolution, but new hordein profiles are not the same as those obtained before cleaning Eventually, chemical cleaning is no longer sufficient and the upper 1-2 mm of the column had to be removed to regain resolution

Work carried out before on barley varietal identification by anion exchange HPLC (Wingad *et al*, 1986, Skeritt *et al*, 1989) seems to have been equally unsuccessful Both Wingad and Skeritt report only initial findings on two and four barley varieties, respectively, and the reproducibility of the obtained hordein profiles was not evaluated The fact that no full study, along the lines of the RP-HPLC reports has been published on barley varietal identification by anion exchange HPLC, since the appearance of the above named preliminary studies, could indicate that the technique was not found to be successful

Experimental evidence on the contamination of anion exchange columns (1 3 2 c) seems to indicate that absorption of polyphenols extracted along with hordeins from wholegrain barley are responsible for the rapid loss of resolution If our conclusion is correct, it should be possible to obtain reproducible hordein elution profiles with white flour, which, because of the removal of husk and bran should, contain no polyphenols The work of Batey (1984) on varietal identification by anion exchange HPLC, proves the point Gliadin elution profiles obtained with white flour were reproducible even after two hundred analyses Unfortunately it would be very impractical to obtain white flour from barley for the purpose of varietal identification alone

It is possible to conclude from the foregoing that anion exchange-HPLC could be used for barley varietal identification if the removal of polyphenols from the hordein extract were possible In the case of RP-HPLC pre-columns are used to remove non-elutable compounds from the injected sample before they reach the column Pre-

columns however are not available for wide pore anion exchange columns, and we were discouraged by the manufacturer (Pharmacia) from trying to make one up from available materials Nevertheless we think it should be possible to find a chromatographic material, which would retain polyphenols without absorbing larger molecules, and make up a pre-column, using the suitable material and commercially available empty pre-column However, considering that the objectives of work on varietal identification, to find a stable column for the identification of Irish barley varieties, and to reduce analysis times without loss in resolution, were achieved by RP-HPLC, further work on anion exchange HPLC was beyond the scope of this thesis

1.4.3 Barley varietal identification and chromatofocusing

It was not possible to obtain standard hordein elution profiles with chromatofocusing, for elution profiles changed with each column cleaning, which was required after every ten injections Therefore in order to use chromatofocusing for varietal identification it will be necessary to obtain in each case, not only the elution profile of the sample, but also that of a number of known varieties until one is found which matches that of the sample Considering that total hordein elution time and column equilibration requires about 90 mins , the need to obtain hordein elution profiles of several known standards along with that of the samples, implies that a single varietal identification could take up to one day

It is difficult to explain why hordein elution profiles change with each column cleaning Since hordein elution profiles are reproducible, before the resolution starts to deteriorate, the changed elution profile after column cleaning cannot be due to denaturation of hordeins or poor repeatability of extraction However, it is possible proteins other than those eluted are extracted from barley with the aqueous buffer and, because their isoelectric point is below the lower limit of the gradient (pH=6), they remain absorbed, and are not fully removed by the recommended cleaning procedures Considering that the partial removal of proteins with low isoelectric points are not uniform processes, the resolving power of the column could change with each cleaning It should be possible to find a cleaning procedure which would remove column contaminants uniformly with each washing, but on account of the obvious drawbacks of chromatofocusing, for varietal identification - (long analysis times, poor resolution) the work was not continued further.

1.5 CONCLUSIONS

RP-HPLC using stable C4 column was found to be a suitable technique for the identification of Irish barley varieties All of the barley varieties examined in the present study could be easily identified from their hordein elution profiles, and these profiles were repeatable, during the lifetime of the column Analysis time could be reduced from 100 to 50 mins without loss of resolution, by using C4 instead of C18 columns Analysis time of 50 mins makes it possible to use RP-HPLC also for the determination of varietal purity on a 20 seeds per sample basis, albeit the total analysis time for a sample of about 17 hrs is still somewhat long It should be possible however to reduce analysis time further by using reverse phase material with shorter than C4 alkyl bonded phase or shorter columns

Anion exchange HPLC is unlikely to contribute to barley varietal identification in its present form While characteristic hordein elution profiles could be obtained for the examined barley varieties with wide pore (500Å) anion exchange columns, repeatability was limited to five or six elutions immediately after column cleaning, after which the resolution deteriorates rapidly Even if a suitable pre-column could be found to improve repeatability, the method would not have significant advantages over RP-HPLC for varietal identification as analysis times at about 45 mins are not much shorter than those obtained by C4 columns However, the resolution is poorer, and the special pumps made of inert materials which must be used with wide pore anion exchange columns cost about twice as much as conventional HPLC pumps

Chromatofocusing was not found to be suitable for routine barley varietal identification The column had to be cleaned after every ten analyses, and hordein elution profiles, while repeatable between column cleanings, changed after each cleaning As no standard hordein profiles could be obtained the only possible role we can see for chromatofocusing in varietal identification, would be to confirm the varietal identity of a sample that has already been identified by other methods

CHAPTER 2

AGRONOMIC AND ENVIRONMENTAL EFFECTS ON ß-GLUCAN LEVELS IN IRISH BARLEY

2.1 INTRODUCTION

2.1.1 Importance of ß-glucans

The polysaccharide (1-3) (1-4)-ß-D-glucan, commonly referred to as ß-glucan, is present in the endosperm of most cereals. In cereal grain the polysaccharide occurs in two forms: water soluble ß-glucan or gum extractable from flour with hot water and insoluble ß-glucan (sometimes referred to as hemicellulose) which can only be extracted with dilute acids or bases or specific enzymes. Both forms have the same chemical structure when extracted, but the latter is believed to be covalently bonded to cell wall proteins (Forrest and Wainwright, 1977). Highest levels of ß-glucans are found in barley and oats, but smaller amounts have also been reported in wheat, rye and triticale (Carr *et al.*, 1990). The levels of ß-glucans in cereals are relatively small when compared to other carbohydrates - they range from 2-10% (w/w) in oats and barley, and are generally below 1% in other cereals (McCleary and Glennie-Holmes, 1985; Carr *et al*, 1990) However, as ß-glucans are water soluble high molecular weight carbohydrates they increase the viscosity of aqueous solutions, and therefore are of importance in brewing, human and animal nutrition

Monogastric animals cannot digest ß-glucan because its chemical structure is similar to cellulose Thus water soluble ß-glucan released from animal feed remains undigested and increases the viscosity of the stomach contents. Increased viscosity will restrict nutrient availability and it may be the reason for the low utilisation of barley when this cereal is included in poultry feed (Åman and Graham, 1987) Insoluble ß-glucans encapsulate nutrients such as starch and intercellular protein in the cell wall and restrict their utilisation (Åman and Graham, 1987)

On the other hand ß-glucans have a decidedly beneficial effect in human nutrition (Wood *et al*, 1991a) As water soluble fibre they help the smooth movement of digested food through the intestines, and facilitate regular bowel movements. In addition, they have a hypocholestemic effect as shown by decreasing serum and low density lipoproteins, and increasing bile acid excretion (Anderson *et al.*, 1984) On account of the importance of the polysaccharide in human nutrition, enrichment of cereal foods with ß-glucans has been proposed (Wood *et al*, 1991a).

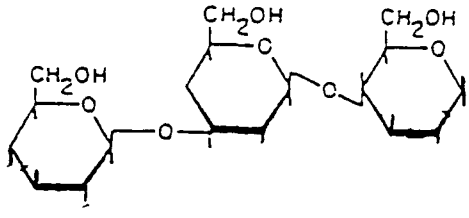
Far more attention has been paid to the adverse effects of ß-glucans in the brewing industry than in nutrition, because ß-glucans are associated with several

problems in the brewing process. High ß-glucan levels derived from adjuncts, like flaked barley, which is added to the malt mash, increase the viscosity of the crude mash extract (wort) and of beer, and retard the filtration rates of both (Bamforth, 1982). Furthermore, the insoluble ß-glucan as a cell wall component is a barrier to the extraction of soluble carbohydrates from the added barley during mashing and thus increased levels of the polymer could lead to decreased brewhouse yields (solid fraction of wort) (Jorgensen and Aastrup, 1988). The presence of the polysaccharide in the final product can cause the appearance of hazy precipitates, which are mainly due to ß-glucans precipitated by alcohol (Bamforth, 1982).

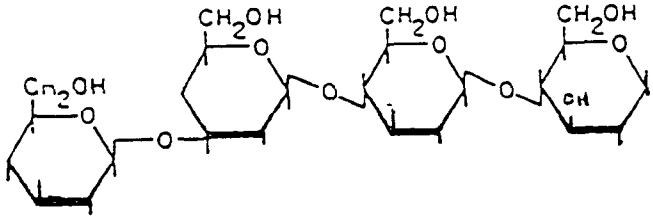
2.1.2 Chemistry of ß-glucans

ß-glucans are high molecular weight polymers of glucose, like starches and cellulose. However, as the physical and chemical properties of ß-glucans are different from the latter two - they were classified as a separate polysaccharide long before their structure had been elucidated. They form viscous solutions with water, whereas starch dissolves normally and cellulose is insoluble Dyes such as Calcofluor, Congo Red and Tiponal form water insoluble starches, ß-glucans can be hydrolysed to glucose with dilute TFA (Wood and Weisz, 1984) but concentrated sulphuric acid is required for the hydrolysis of cellulose (Goldstein, 1981)

Early studies predicted structural similarities between ß-glucan and cellulose (Meredith *et al* , 1955) as both could be degraded to the disaccharide, cellobiose Later it was shown that ß-glucan could be hydrolysed by cellulase from *Streptomyces* sp QM B814 (Parrish *et al.*, 1960) to a mixture of mainly tri- and tetra-saccharides with the following structures

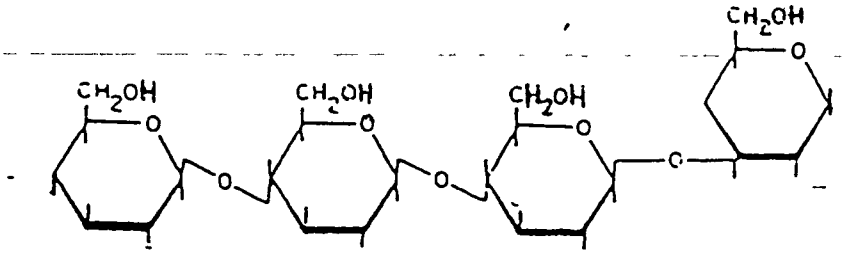


4-0-ß-laminarinbiosyl - D - glucose

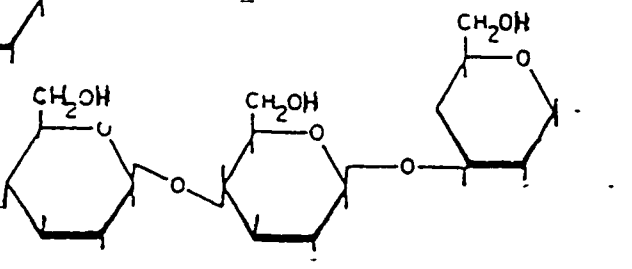


4-O-β-laminarinbiosyl - D -cellobiose

The polysaccharide could be also hydrolysed by laminarinase, an enzyme specific to ß 1-4 linkage on ß 3-1 linked glucose, to yield the tri-and tetrasaccharides below:



3-O-β-D-cellotriosyl-D-glucose



3-O-β-D-cellobiosyl-D-glucose

It could be concluded from the four oligomers obtained by enzymic hydrolysis that ß-glucan consisted of cellotriosyl and cellotetrosyl units linked by ß(1-3) bonds. The isolated nature of the ß(1-3) bonds was confirmed later by C13 NMR (Dais and Perlin, 1982). Larger ß(1-4) units have also been identified (Wood *et al.*, 1991b), but they occur only in small amounts relative to the triosyl and tetrasyl units.

Structural differences between oats, barley and lichenan (Wood *et al.*, 1991b) ß-glucans were determined from HPLC oligomer patterns obtained after hydrolysis with lichenase Oat ß-glucans have twice as many cellotriosyl as cellotetrasyl units, in barley this ratio increases to about three, and in lichenan to twenty. Each ß-glucan also contains a small fraction cellopentosyl to cellootasyl units It is not clear at present, however, how the different tri- to tetrasaccharide ratios effect the physical properties of the particular ß-glucan.

The biochemical behaviour of ß-glucans reflects their structural characteristics. The (1-3) acid (1-4) linkages between the glucose units are all ß like in cellulose, hence they can be hydrolysed completely to glucose by cellulase form *Trichoderma resii* (Martin and Bamforth, 1980) or *Penicillium funicolosum* (Bamforth, 1983). However, on account of the solubility of ß-glucan in water glucose linkages are more accessible to the enzyme, and hydrolysis is much faster than with cellulose (Hartley, 1983). Furthermore, the (1-4) linkage on the (1-3) (1-4) linked glucose can be selectively hydrolysed to yield a mixture of 3-0-ß-cellotriosyl- and 3-0-ß-cellobiosyl-D-glucose oligomers by lichenase or ß-glucanase which are inactive on both starch and cellulose. Analytical methods for the determination of ß-glucan make use of the selectivity of the

foregoing enzymatic hydrolyses

2.1.3 Analytical methods for the determination of ß-glucans

2.1.3a Physical and chemical methods

One of the earliest methods for the determination of ß-glucans is based on the viscosity of aqueous barley extract (Greenberg and Whitmore, 1974). It is assumed by the authors that changes in viscosity are entirely due to changes in ß-glucan levels, and other co-extractants such as starches and pentosans have only minimal effect. While the assumption might not be entirely correct viscosity shows a remarkably good correlation with ß-glucan levels determined by the difference method (Jorgensen and Aastrup, 1988). The method is very fast and simple, and it has been used successfully as a screening technique in barley breeding programmes (Jorgensen and Aastrup, 1988).

Another early method is based on the difference between starch glucose and total carbohydrate glucose in an aqueous extract of barley flour (Wood *et al.*, 1977) Starch is selectively hydrolysed to glucose by commercial amylase and non-starch carbohydrates with dilute sulphuric acid The authors assumed that all non-starch carbohydrates are ß-glucans, hence the difference between starch and total glucose corresponds to glucose released by ß-glucans. One of the disadvantages of the method is that along with starch and ß-glucans other polysaccharides, such as pentosans (approx. 20% glucose), can also be extracted and hydrolysed under the conditions employed. Furthermore commercial amylase often contains ß-glucan degrading enzymes

In order to avoid interferences from extracted starch, an analytical method based on the selective precipitation of ß-glucan by Calcofluor, a fluorescent dye, was developed (Wood and Weisz, 1984) The precipitated Calcofluor ß-glucan complex is isolated, the ß-glucan is hydrolysed with dilute TFA and glucose is determined by HPLC. ß-glucan levels determined by selective precipitation compare well with those determined by the difference method Disadvantages of the assay, based on ß-glucan precipitation, are the time consuming purifications of both the Calcofluor complex and the hydrolysate In addition, starchy material can be included in the precipitate which will result in over-estimation of ß-glucan levels

A simple variation of the Calcofluor technique is the staining of barley flour with Calcoflour and measuring fluorescence emission of the flour suspension in glycerol (Jorgensen and Aastrup, 1988) A reasonable correlation was obtained between

fluorescence intensity and ß-glucan levels determined later by enzymatic methods. As it is unlikely that all ß-glucan in the cell is stained, the method is probably not accurate for the determination of total ß-glucans. It is, however, a very convenient way of determining if a barley sample has low or high ß-glucan levels.

A fast and quantitative method for the determination of ß-glucan levels with Calcoflour makes use of the increase of fluorescence when the dye is added to an aqueous solution of ß-glucan (Wood *et al.*, 1982). Unfortunately Calcofluor m dilute solution is very sensitive to light and the fluorescence emission is unstable. The problem can be overcome by using a flow injection device - where the reagent is mixed with the sample in a dark coil and injected into the flow-cell without being exposed to light. The method is fast and precise and can be automated A very good correlation is obtained between the flow injection method and a more ß-glucan-specific enzymic method (Munck *et al* , 1989) The use of Calcoflour m post column derivatisation for the detection of ß-glucans separated by site exclusion chromatography has been also reported (Suorti, 1993)

2.1.3b Enzymic methods

As ß-glucans are water soluble they can be fully and/or partially hydrolysed by several enzymes Of these, cellulase, lichenase, ß-glucanase and ß-glucosidase have been made use of in the different methods of ß-glucan analysis. Work on the development of a precise enzymic method for the determination of ß-glucans concentrated on finding enzymes which hydrolyse ß-glucans quantitatively, and on ensuring that these do not contain impurities which release glucose from co-extracted starch

The first enzyme based analytical methods used cellulase for selective hydrolysis of the analyte because of the structural similarities between cellulose and ß-glucan. Cellulase preparations from different fungal sources were examined. Cellulase from *Trichoderma resii* (Martin and Bamforth, 1980) *Penicillium funiculosum* (Bamforth, 1983) gave complete hydrolysis of ß-glucan to glucose Unfortunately, however, all the suitable preparations of cellulase contained varying amounts of amylase which releases glucose from co-extracted starch and results in over-estimation of ß-glucan. In order to eliminate interference from co-extracted starch ß-glucan was removed from the crude extract, or amylase was removed from commercial cellulose preparations. In a method reported by Åman and Graham (1987), starch in the crude barley extract was hydrolysed

to glucose by amylase and ß-glucan was precipitated with aqueous ethanol. On account of the solubility of glucose in aqueous ethanol, the precipitated ß-glucan could be obtained free of glucose. Glucose was then released from the isolated ß-glucan by using a crude ß-glucanase preparation from *Aspergillus niger*, as residual amylatic activity was not a problem. Isolation of ß-glucans from oat extract by precipitation with isopropanol followed by purification and hydrolysis to glucose with commercial cellulase was also reported (Dawkins and Nnanna, 1993). Another approach was the selective de-activation of amylase by heating to 70°C (Bamforth, 1983). This method was successful for the de-activation of amylase in cellulase derived from *Penicillium funiculosum*, but the enzyme derived from *Trichoderma reesi* lost most of its cellulase activity (Martin and Bamforth, 1980) upon purification.

Both analytical methods, the use of crude ß-glucanase on isolated ß-glucans, and the use of heat-treated cellulase gave precise ß-glucan levels with the coefficients of variation ranging from 3 to 7 % However, as they were not compared their relative accuracy is not known

While the assays based on cellulase hydrolysis can be used for relatively precise determination of ß-glucan levels, the methods are not without drawbacks Amylase-free cellulase is not available commercially and each batch needs to be heat-treated, purified and tested for amylase and ß-glucan activity The purified cellulase is not very stable It lasts only one week in solution at 4°C, and only seven months freeze dried and stored at -20°C. (Bamforth, 1981, Carr *et al* , 1990). If stable commercial ß-glucanase is used ß-glucan needs to be isolated free of glucose which is a tedious and time consuming procedure (Åman and Graham, 1987) Finally, hydrolysis of ß-glucan by cellulase or ß-glucanase takes about 3 hours which limits the number of samples that can be analyzed per day (Carr *et al* , 1990)

In order to reduce assay time and eliminate the inconvenience of enzyme testing and purification a second enzymic procedure based on faster and more stable enzymes was devised (Anderson *et al* , 1978, McCleary and Codd, 1991). The assay is based on the depolymerisation of ß-glucan by lichenase which only cleaves the (1-4)-ß linkages of 3-substituted-D-glucopyranosyl residues Hence, it is very specific to ß-glucan The resulting cellotriosyl and cellotetrasyl units are hydrolysed by ß-glucosidase to glucose Both enzymes are commercially available free of amylase, and retain their activity indefinitely if stored at -20°C Both enzymes are fast acting - the two hydrolyses are

completed in 30 minutes.

2.1.4 Objectives

While there are a wide range of assays reported for the determination of ß-glucans, the only published comparative study is that of the lichenase and the FIA assays. Therefore, the first objective of the present work on ß-glucans, is to evaluate the various assays, in order to establish which is the most suitable for routine determination of ß-glucans within normal laboratory facilities The second objective is to use the found assay for the evaluation of the influence of varietal, agronomic and environmental factors on the ß-glucan levels of barley grown in Ireland. Some work has been carried out on agronomic factors influencing ß-glucans levels (Bendelow, 1978 Molino and Conde, 1982) but the barley used in the study was grown in different climates and the results might not apply here

2.2 MATERIALS AND METHODS

2.2.1 Determination of (1-3)(1-4)-ß-D-glucan in barley using the lichenase method

2.2.1a Materials and general procedures

Barley (1-3), (1-4) ß-glucan was supplied by Sigma U.K Ltd. Lichenase (EC 3 2 1 73) (McCleary, 1988a) and ß-glucosidase (EC 3 2.1 21) (McCleary and Harrington, 1988b) were supplied by Megazyme (Aust) Pty Ltd North Rocks, NSW Glucose was measured by a kit also supplied by Megazyme, which is based on glucose oxidase-peroxidase (Gopod) technique, originally reported by Trinder, (1969) Other references on the Gopod Technique are Blakeney and Matheson, (1984), McCleary and Codd, (1991)

2.2.1b Preparation of buffers and enzymes

All reagents used were of analytical grade

Sodium phosphate buffer (20 mM, pH 6 5) Sodium dihydrogen orthophosphate dihydrate (3 12 g) is dissolved in 900 ml of distilled water and pH adjusted to 6 5 by addition of 0 1 M sodium hydroxide (£ 50 ml) The volume is then adjusted to 1 litre with distilled water.

Sodium acetate buffer (200 mM, pH 4 0) Sodium acetate trihydrate (4 8 g) is dissolved in 990 ml of distilled water and 9 6 ml of concentrated acetic acid is added. The pH

was adjusted to 4 0 with 0 1 M HCl Samples of this buffer are diluted four-fold with distilled water

Lichenase (50 U ml^{-1}) The entire contents of one vial of Lichenase (supplied by Megazyme (Australia) Pty Ltd) is diluted to 20.0 ml with 20 mm sodium phosphate buffer (pH 6 5) The enzyme solution is divided into 5 ml aliquots and stored frozen in plastic vials

ß-glucosidase (2 U ml^{-1}) The entire contents of one vial of ß-glucosidase (supplied by Megazyme (Australia) Pty Ltd) is diluted to 20 0 ml with 50 mM sodium acetate buffer (pH 4.0). The enzyme solution is divided into 5 ml aliquots and stored frozen in scintillation vials.

Glucose reagent buffer Concentrated buffer is prepared by dissolving mono-potassium orthophosphate (136.0 g), sodium hydroxide (32 0 g) and 3-hydroxybenzoic acid (15 0 g) in 900 ml of distilled water by stirring at room temperature. The pH is adjusted to 7 4 using 2 M hydrochloric acid, the volume is adjusted to 1 litre and sodium azide (2 0 g) is added This buffer is stored in dark containers or bottles at 4°C and is stable up to 12 months Working buffer is prepared by diluting aliquots of this solution ten-fold with distilled water

Note Sodium azide should not be added to solutions of low pH because it results in the formation of poisonous gas

Glucose oxidase-peroxidase-4-amino-antipyrine reagent (Gopod) The contents of one vial of Gopod reagent (supplied by Megazyme (Australia) Pty Ltd as a kit for glucose determination) is dissolved in 1 litre of glucose reagent working buffer solution This reagent is stored at 4°C and on ice during use Cold reagent is added to assay tubes and then incubated at 50°C for 30 minutes

2.2.1c Methodology of ß-glucan determination

Barley corns are finely ground through a 0 5 mm screen (Tecator cyclone grinder or equivalent) 120 mg of flour is weighed and added to a 12 ml pyrex test tube. The flour is wetted with 0 2 ml of an aqueous ethanol solution (50/50 v/v) to aid dispersion of the flour sample Sodium phosphate buffer (20 mM, pH 6 45, 4 0 ml) is added to the test tube and contents stirred vigorously on a vortex mixer. The samples are incubated in a boiling water bath for 60 seconds. The tubes are removed and vigorously mixed on a vortex and re-incubated at 100°C for a further two minutes and again stirred. The

samples are moved to a water bath at 50°C and allowed to equilibrate for 5 minutes. Lichenase (0 2 ml, 10 µ) is added with stirring, the samples are then incubated for 1 hour at 50°C with occasional stirring. Sodium acetate buffer (200 mM, 5 ml) is added to the samples to terminate the lichenase activity The tubes are sealed with Parafilm^R and the contents are mixed by inversion several times Once the Parafilm^R has been removed the samples are allowed to equilibrate to room temperature for 5 minutes. The tubes are centrifuged at 3,000 rpm for 10 minutes and 0.1 ml samples carefully transferred to bottoms of three test tubes (12 ml capacity) using a Finn pipette (Lab systems OY, Finland) 0 1 ml ß-glucosidase (0.2 U) in 50 mM acetate buffer (pH 4.0) is added to two of these test tubes and to the third, a blank, 50 mM acetate buffer (0.1 ml, pH 4 0) is added All tubes, inclusive of blanks and controls are incubated for 20 minutes at 50°C After incubation 0 1 ml of ß-glucosidase-supernatant is mixed in 10 ml test tubes with Gopod reagent (3 ml) and incubated at 50°C for a further 20 minutes. After 20 minutes the test tubes are removed from the water-bath and absorbance of samples is read at 510 nm within an hour

2.2.1d Calculations

$$\text{ß-glucan (\% W/W) as is} = \Delta E \times F \times 94 \times \frac{1}{1000} \times \frac{100}{W} \times \frac{162}{180}$$

$$= \Delta E \times \frac{F}{W} \times 8\,46$$

Where ΔE = absorbance after ß-glucosidase treatment minus blank absorbance; F = a factor for the conversion of absorbance values to µg glucose = 100/absorbance for 100 µg of glucose], 94 = volume correction factor (0 1 ml out of 9 4 ml was analyzed), 1/1000 = conversion from micrograms to milligrams, W = weight of sample analyzed, 100/w converts back to 100 µg of sample, 162/180 = a factor to convert from free glucose, as determined, to anyhydroglucose, as occurs in ß-glucan In order to express ß-glucan levels on dry weight basis moisture contents were determined by drying 5 g ground samples in metal petri dishes at 130°C for 1 h

2.2.2 Enzymic determination of ß-glucan using cellulase

2.2.2a Materials and general procedures

Barley (1-3) (1-4) ß-glucan standard and cellulase (EC 3 2 14) from *Penicillium funiculosum* were supplied by Sigma Chemical Co. Ltd. Dorset. Glucose was determined by a commercial kit based on the glucose oxidase Peroxidase (Gopod)

techniques and supplied by Megazyme (Aust) Pty. Ltd. North Rocks, NSW

2.2.2b Preparation of buffers and enzymes

All reagents used were of analytical grade

Sodium acetate buffer· 34 02 g of sodium acetate trihydrate was dissolved in 4 5 litres of distilled water. The pH was adjusted to 4 0 using 1 M HCl. The volume was adjusted to 5 litres, after the correct pH had been obtained. This resulted in a 0.05 M sodium acetate-HCl dialysis buffer.

Sodium succinate buffer· 13.51 g of sodium succinate was dissolved in 900 ml with 0.2% (w/v) sodium azide. The pH was adjusted to 5 5 by addition of 1 M HCl and the volume made up to 1 L with distilled water and was stored at 4°C.

Enzyme preparation. 0 4 g of commercial cellulase (Sigma C0910) from *P funiculosum* was transferred into a 12 ml pyrex test tube and suspended in 10 ml 0.05 M sodium acetate -HCl buffer (pH 4 0) for 10 minutes and then centrifuged for 10 minutes at 2,500 r p m. The supernatant was transferred to 5 test tubes (12 ml pyrex tubes) and heated to 70°C in a water bath for 1 hour with subsequent cooling in an ice bath for 2 minutes This heat-treated supernatant was dialysed against 2 l of 0 05 M sodium acetate-HCl buffer at 4°C for 16 hours. After the dialysis step, the solution was centrifuged for 30 minutes at 2,500-3,000 r p m The supernatant which contained the purified cellulase was collected and it could be stored at 4°C for up to one week The purified enzyme could be stored up to seven months freeze dried at -20°C.

2.2.2c Methodology of β-glucan determination

Extraction of soluble β-glucan 200 mg of barley flour was weighed into a 12 ml pyrex test tube, and refluxed twice with 80% (v/v) ethanol (5 ml) for 0 5 hours each After each reflux the mixture was centrifuged and the supernatants discarded Water soluble β-glucan was extracted from the ethanol-treated residues with water (10 ml) for 1 hour at 100°C, the extracts were centrifuged to remove insoluble material, and the supernatants were collected The residues were washed with an additional 10 ml of water and centrifuged, the two supernatants were combined, and the final volume was adjusted to 25 ml with water

Extraction of total β-glucan 200 mg of barley flour was weighed into a 12 ml pyrex

test tube Samples were refluxed twice in 80% (v/v) ethanol (5 ml) for 0.5 hours each, centrifuged and the supernatants was discarded in each case. Total β-glucan was extracted from ethanol treated residues with 1 M sodium hydroxide solution (10 ml) at 20°C for 16 hours. The mixture was neutralized with 1 M HCl, centrifuged to remove insoluble material and supernatants were collected The residues were washed with 10 ml water, the mixture was centrifuged. The supernatant from the washing was combined with the neutralized extract, and the final volume was adjusted to 25 ml.

Determination of β-*glucan.* 0 4 ml of supernatant was transferred to two 12 ml pyrex test tubes. Sodium succinate buffer (0 4 ml, 0.05 M, pH 5 5) was added to each test tube. The prepared enzyme solution (0 2 ml) was added and all samples inclusive of blanks and control, and samples were incubated for 3 hours at 40°C 0.1 ml of the solution (enzyme, substrate and product) was transferred to new test tubes. 3 ml of Gopod reagent was added, followed by incubation at 50°C for 20 minutes and cooling to room temperature Absorbances were read at 510 nm

Note Enzyme and reagent blanks as well as a ß-glucan standard were prepared with every test series This ensures that the Gopod does not erroneously produce colour for reagents and also shows that the prepared enzyme has sufficient activity to hydrolyse extracted β-glucans

2.2.2d Calculations

$$\text{ß-glucan (\% w/w as is)} = \frac{A_1}{A_1} \times 100 \times 625 \times \frac{1}{1000} \times \frac{100}{W} \times \frac{162}{180}$$

Where A_1 and A_2 = absorbance at 510 nm of 100 µg glucose and of sample extract after cellulase hydrolysis, 100 = conversion of absorance ratio to µg of glucose in treated sample extract, 625 = volume correction factor (0 4 ml out of 25 ml followed by 0 1 ml out of 1 ml, i e 0 04 ml out of 25 ml),

$\frac{1}{1000}$ = conversion from micrograms to milligrams,

$\frac{100}{W}$ = conversion back to 100 g sample,

W = weight of sample analysed,

$\frac{162}{180}$ = a factor to convert from free glucose to anhydro glucose as occurs in ß-glucan.

In order to express ß-glucan levels on dry weight basis moisture contents were determined by drying 5 g ground samples in metal petri dishses at 130°C for 1 hour.

2.2.3 Field trials and procurement of samples

A series of trials to examine the potential of autumn-sown spring malting barley vis a vis true winter barley were carried out in 1990-1992 on sites in Carlow, South Kilkenny and mid-Wexford Trial design was a randomised block split-plot with six replications. The cultivars Blenheim, Puffin and Pastoral were sown at three sowing dates in each site at seeding rates of 160, 175 and 190 kg/ha. The level of fertiliser applied varied from 50 -130 kg N/ha The level of fertiliser N was applied in two splits (1st application at growth stage 26, 2nd application at growth stage 30) Stem and foliar diseases were controlled using a three fungicide spray programme At maturity the plots were harvested using a modified combine harvester, and grain yields, moisture, protein and other grain quality parameters assessed. These samples were used for examining the effect of genotype and environment interaction in levels of β-glucan and β-glucanase in malting barley

2.3 RESULTS AND DISCUSSION

2.3.1 Evaluation of analytical methods

In order to study the effects of external factors on barley ß-glucan levels it was necessary to find a suitable method for the determination of the polysaccharide. The preceding section showed that there are a wide range of methods available, however, independent evaluation of the various assays has not been reported so far Consequently a method of evaluation had to be carried out in order to establish which of the many methods is most suitable for the purposes of the present study

As variation between the ß-glucan levels of barley samples taken from the same plot is expected, at least six samples had to be analysed from each plot to ensure that differences due to external factors are statistically significant. Consequently about 180 samples were analysed in the present study Therefore, the method for the determination of β-glucans needs to be fast and relatively simple - to avoid errors that could arise from extensive handling The particular method chosen will also need to be precise to detect small differences in β-glucan levels

Precisions expressed as coefficients of variations of the methods reported since 1980 are shown in Table 1 According to the listed values reported precisions for barley range, from 2 6 to 7 1 %, and lower values have been reported for other commodities.

However, some of these methods are time consuming and unsuitable for the analysis of a large number of samples Precipitation of ß-glucans by Calcofluor (Wood and Weisz, 1984) with ethanol (Hadden and Graham, 1987), followed by washing, are relatively time consuming procedures and there is always the danger of residual glucose or starch in the precipitate. The flow injection method for the determination of ß-glucan is fast and precise, but the necessary instrumentation was not available Consequently only the cellulase assay as modified by Carr *et al*, (1991) and the lichenase assay were evaluated (McCleary and Codd, 1991)

Both methods have drawbacks and advantages - and it is not possible to decide which is most suitable for the present study without comparative determinations. The cellulase method is slower, but it involves fewer steps than the lichenase method. Hence random errors are less likely to occur, an important consideration when a large number of samples are being analysed

However, each batch of commercial cellulase needs to be purified and tested for contaminating amylolytic activity and adequate β-glucanase activity Residual amylase can release extra glucose from co-extracted starches which will lead to the over estimation of β-glucan levels On the other hand the lichenase assay is faster, the required enzymes can be obtained in a purified form, but extensive sample handling could be a drawback

The two methods were evaluated by carrying out repeated determinations of ß-glucan levels in eight Irish barley varieties, and the ß-glucan levels obtained are shown in Table 2 Both total and water-soluble ß-glucans were determined by the cellulase method but only total ß-glucans by the lichenase method It should be possible to determine water-soluble ß-glucans by the lichenase method also, but because these are thought to be of little importance in brewing the correct experimental procedure has not been worked out. Precisions of β-glucan levels determined by the lichenase assay as expressed by coefficients of variation are within the range of reported values (McCleary and Codd,1991) (Table 2) for barley varieties Blenheim, Chef, and Corniche but somewhat higher for Chad, Digger Ashling, Grit and Prisma The coefficients of variation of the cellulase assay, however, are considerably higher for most barley varieties than reported by Carr *et al.*, (1991) It must be pointed out that the reported precision for ß-glucan levels in barley is based on the analysis of one sample in triplicate which might not be representative of the repeatability of the assay. Precisions

obtained by the same author for a variety of oat products are more in line with our findings.

The results (Table 2) indicate that there is a very poor agreement between the cellulase and lichenase assays. Total ß-glucan levels obtained by the cellulase assay are considerably higher than those of soluble ß-glucans, which is expected. However, there is a very large difference between the total ß-glucan levels as determined by the lichenase and the cellulase assays, the latter being much higher in most cases. On the other hand total ß-glucan levels obtained by the lichenase assay are higher than water soluble ß-glucans obtained by the cellulase assay, albeit the difference is considerably smaller than between the two total ß-glucan levels

At first glance it could be argued that much of the bound ß-glucan is inaccessible to lichenase, hence the higher readings with 24 hours dilute sodium hydroxide extraction Opinions about the under-estimation by the lichenase assay have been expressed before in literature (Henry and Blakeney, 1988), although without proof Removal of some bound ß-glucan by lichenase could explain the higher levels obtained with lichenase than with aqueous extraction While the foregoing explanation might sound plausible it is contradicted by the following experimental evidence

Considering that the water insoluble fraction of ß-glucan is bound to protein addition of non-specific protease to lichenase, and incubation under conditions required for complete protein hydrolysis should release some of the bound β-glucans Similarly, if dilute sodium hydroxide solution releases all bound β-glucans at room temperature in 24 hours, a brief boiling with the same solution should release at least some of the bound polysaccharide However, neither method of extraction caused a significant increase in β-glucan levels above that obtained by normal lichenase extraction (McCleary and Glennie-Holmes, 1985) Furthermore, the lichenase assay shows very good agreement with the Calcoflour fluorescence assay which is based on dilute sulphuric acid extraction at 100°C

While the evidence available (McCleary and Glennie-Holmes, 1985), indicates that the lichenase assay is probably more accurate for the determination of ß-glucans, it does not provide an explanation for the higher levels obtained with the cellulase assay If ß-glucan is not the source of extra glucose it must originate either from grain starch or husk hemicellulose There is no evidence to indicate that dilute sodium hydroxide hydrolyses starch at room temperature, but it does dissolve the hemicellulose (approx

20 % glucose) in grass or straw (Al-Katrib *et al*, 1988) and the husk has similar composition. It is also possible that commercial cellulase also contains some hemicellulase, as they are usually both present in the same microorganism; thus glucose could be released during incubation of the sodium hydroxide extract with cellulase However, according to our calculations based on 15% husk in grain (Brigg, 1978), 30 % (w/w) hemicellulose in the husk (Goldstein, 1981) and 20 % (w/w) glucose in hemicellulose (A. Frohlich and Burke, 1991), glucose from hemicellulose, could add up to 1% (w/w) to the determined ß-glucan level. While the foregoing might explain some of the differences between total ß-glucan levels obtained by the lichenase and the cellulase assays, larger differences, as in the case of varieties Blenheim, Corniche and Prisma, cannot be attributed entirely to glucose from hemicellulose

The foregoing arguments indicate that it is not possible to decide with absolute certainty which of the two assays studied here is more accurate. However, the lichenase assay is the recommended method of the Australian Chemical Institute (McCleary and Codd, 1991) the European Brewery Convention (Munck *et al*, 1989), and the Association of Official Analytical Chemists (Zygmunt and Paisley, 1993) it has been subjected to inter-laboratory tests, and it is the most widely used method at present for ß-glucan determinations Consequently, if we wish to compare ß-glucan levels found in our study to those obtained by other authors, it is more advisable to use a widely accepted assay

2.3.2 Effects of external factors on ß-glucan levels

2.3.2a Varietal effects

Barley samples used to evaluate varietal effects on ß-glucan levels were obtained from three different trials The first two trials were sown in the Spring of 1990 and 1991 by Teagasc at Oak Park, and they involved eight and six varieties, respectively. The third trial was carried out by Teagasc in 1992, with the objective to compare Autumn sown malting barleys with feed barleys at three different locations, and at varying levels of applied nitrogen

ß-glucan levels of the different malting barley varieties of the foregoing trials, along with expected precision - as expressed by the coefficient of variation are shown in Tables 3, 4 and 5. The tabulated values of the 1990 trials represent an average of sixteen determinations of bulked samples composed of barleys from six different plots, whereas those of the 1991 trials are an average of individual ß-glucan levels each

Table 1: Reported co-efficients of variation of ß-glucan levels in barley

Authors	Assay	Year	Reported % CV
Wood and Weisz	ß-glucan pptn acid hydrolysis	1984	4.7
Carr *et al.*	Cellulase hydrolysis	1990	3 1
Bamfort	Cellulase hydrolysis	1983	7 0 - 7 1
Åman and Graham	ß-glucan pptn, ß-glucanase hydrolysis	1987	1.9 - 3.2
McCleary and Codd	Lichenase and ß-glucanase hydrolysis	1991	2.0 - 5.0

Table 2: ß-glucan levels in barley varieties obtained by the lichenase and cellulose assays

Variety	Total ß-glucan (w/w)		Δ	Water soluble ß-glucan cellulase assay	Differences between total and soluble ß-glucans	
	Lichenase assay	Cellulase assay			Cellulase assay	Lichenase assay
Blenheim	2 98 ±0 16 (5 4)	5 34 ±0 72 (13 5)	2 36	2 17 ±0 19 (8 7)	3.17	0 81
Chad	3 41 ±0 39 (11 4)	3 53 ±0 02 (0 5)	0 12	2 99 ±0 46 (15 0)	0 54	0 42
Chef	2 46 ±0 10 (4 0)	3 44 ±0 13 (3 8)	0 98	2 31 ±0 20 (8 7)	1 13	0 15
Digger	3 36 ±0 26 (7 7)	3 82 ±0 40 (10 4)	0 46	3 82 ±0 40 (10 5)	0 0	-0 46
Aisling	3 02 ±0 24 (7 9)	3 87 ±0 37 (9 6)	0 85	2 64 ±0 41 (15 5)	1 23	0 38
Grit	2 60 ±0 25 (9 6)	3 52 ±0 16 (4 6)	0 92	3 52 ±0 16 (4 5)	0 0	-0 90
Corniche	2 28 ±0 09 (3 9)	3 63 ±0 23 (6 3)	1 35	2 22 ±0 0 (0)	1 41	0 06
Prisma	2 41 ±0 18 (7 5)	4 66 ±0 66 (14 0)	2 25	1 81 ±0 21 (11 6)	2 85	0 6
No of repeats	16	6		6		

( ) = co-efficients of variations, Δ = difference between total ß-glucan levels determined by the lichenase and cellulase assays

corresponding to six samples obtained from a different plot

The coefficients of variation of ß-glucan levels of the eight and six variety trials (Tables 3 and 4), are about the same, thus indicating that variation between plots in this case is no larger than the error of determination. Good agreement between the two coefficients of variation is probably due to the uniform soil at the location of the six variety trials However, when the soil at the trial location is less uniform, as in the case of the three variety trials (Table 5), the variation between plot ß-glucan levels is nearly twice as high as the error of determination.

The range of ß-glucan levels 2 28 - 3.41% for the eight varieties and 1.59 - 2.16% for the six variety trials (Table 5) is evidence for a strong varietal effect. Varietal effect is confirmed by the statistical significance of the differences between the ß-glucan levels of the studied varieties Differences between consecutive ß-glucan levels from the eight and the six variety trials, and the statistical significance of these, are shown in Tables 6 and 7 Among the eight varieties of the first trial five ß-glucan levels are statistically significant, and the differences between Prisma and Chef, Blenheim and Aisling, Digger and Chad were not significant (Table 6) Similarly, among the six varieties of the second trial three ß-glucan levels had statistical significance, and difference between Grit, Alexis and Blenheim, Aisling and Chad were not significant (Table 7) Differences between non-consecutive values in each trial are significant in most cases of the 99% confidence level, thus giving four highly significant ß-glucan levels in the first trial and three in the second trial. The foregoing statistical analysis confirms varietal influence on ß-glucan levels in malting barley

Varieties common to the two trials, namely Grit, Blenheim, Aisling and Chad have 0 84 -1 25% higher ß-glucan levels in the eight variety trials, although the relative order of values is the same in both (Table 8) Considering that the two sets of barleys were grown in different years and locations, factors other than variety must influence ß-glucan levels. Similar observation was made with Canadian barleys (Bendelow, 1975) and the ß-glucan levels of twelve different varieties varied considerably between three locations, but the relative order of values remained unchanged

The influence of factors other than variety on obtained ß-glucan levels is also shown in the trial which compares feed barley with malting barley in different areas at three rates of applied nitrogen (Table 5) There is no difference between the ß-glucan levels of Blenheim (malting barley), Puffin and Pastoral (feed barley) at low

Table 3: Effect of variety on ß-glucan levels of barley grown in 1990

Variety	Corniche	Prisma	Chef	Grit	Blenheim	Aisling	Digger	Chad
ß-glucan level (% w/w) n=20	2.28	2.41	2.46	2.60	2.96	3.02	3.36	3.41
s.d.	0.09	0.18	0.10	0.25	0.16	0.24	0.26	0.39
CV %	3.9	7.5	4.0	9.6	5.4	7.9	7.7	11.4

Table 4: Effect of variety on ß-glucan levels of barley grown in 1991

Variety	Teal	Alexis	Grit	Blenheim	Aisling	Chad
ß-glucan level (w/w) n = 6	1.59	1.75	1.76	1.88	2.07	2.16
s.d.	0.13	0.10	0.061	0.14	0.11	0.21
CV %	8.2	5.7	3.5	7.4	5.3	9.7

Table 5: Effect of variety on ß-glucan levels, feed and malting barley

Location	Oak Park			Kildalton			Gusserane		
Variety	Bl	Pa	Pu	Bl	Pa	Pu	Bl	Pa	Pu
Low N									
ß-glucan level (% w/w) n = 6	1.69	1.51	1.40	2.10	2.09	1.86	1.58	1.34	1.40
s.d.	0.37	0.21	0.33	0.57	0.08	0.25	0.18	0.25	0.15
CV %	24	15	19.5	27	3.8	13.4	11.3	18.6	10.7
Normal N									
ß-glucan level	2.20	1.63	1.53	2.22	2.15	1.74	1.50	1.47	1.30
s.d.	0.15	0.33	0.45	0.08	0.13	0.28	0.41	0.15	0.28
CV %	6.8	20.2	29.4	3.6	6.0	16.1	27.3	10.2	21.5
High N									
ß-glucan level	2.00	1.56	1.56	2.22	2.17	1.85	1.28	1.38	1.40
s.d.	0.15	0.38	0.28	0.15	0.14	0.14	0.13	0.19	0.34
CV %	7.5	24.4	17.9	6.8	6.5	7.6	10.2	13.8	24.3

Bl = Blenheim; Pa = Pastoral; Pu = Puffin; N = level of applied nitrogen levels

of applied nitrogen - or at Gusserane at any level. However, as the rate of applied nitrogen increases at Oak Park, values for Blenheim become higher than those of the two varieties of feed barley. Similarly, at Kildalton there is no difference between the ß-glucan levels of the three varieties at the low rate of applied nitrogen, but at medium and high rates both Blenheim and Pastoral have higher levels than Puffin. The aforementioned differences are statistically significant at the 95% confidence level (Table 9). The results obtained here indicate that there is a definite varietal effect on the ß-glucan levels of barley. However, the observed differences between the ß-glucan levels of varieties are not constant and can change with changing environmental factors (location, year of sowing) and agronomy (rates of applied nitrogen, time of sowing). The influence of external factors on ß-glucan levels will be examined in the subsequent sections.

2.3.2b Sowing dates

It has been reported before that in a Mediterranean environment sowing dates had a significant effect on malting barley ß-glucan levels (Molino-Cano and Conde, 1982), and later sowing resulted in higher levels. In order to evaluate the effect of sowing date under Irish conditions, trials were carried out at Kildalton and at Oak Park. At each site six plots of malting barley variety, Blenheim, were sown in the Autumn of 1991, on three different dates separated by three weeks, and seeding rates and nitrogen levels were normal. Barley samples were taken from the six plots corresponding to each sowing date, and the listed ß-glucan levels represent an average of the six plot values.

β-glucan levels obtained for barley at different sowing dates along with relevant statistical parameters are shown in Table 10. Coefficients of variation of the Kildalton values are the same as that of the assay - but two of the Oak Park values are considerably higher, probably because the soils at Oak Park are less uniform, and there are greater differences between plots. The reported increase in ß-glucan levels with later sowing dates based on Spanish trials (Molino-Cano and Conde, 1982) could not be observed here. In fact the small differences between the three different sowing dates at the two sites were not statistically significant (Table 11). Therefore, according to the data obtained in the present work, Autumn sowing dates have no effect on the ß-glucan levels of malting barley under Irish growing conditions. One possible reason for the increase of ß-glucan levels in a Mediterranean environment

Table 6: Differences between ß-glucan levels due to varietal influences - 1990 barley samples

Variety vs Variety	Difference	F-value	Significance
Cornche-Prisma	0.13	6.11	*
Cornche-Chef	0.18	25.23	**
Prisma-Chef	0.05	0.41	n.s.
Prisma-Grit	0 19	5.89	*
Chef-Grit	0 14	5 4	*
Chef-Blenheim	0 50	99 0	**
Grit-Blenheim	0 36	18.2	**
Grit-Aisling	0.42	21 7	**
Blenheim-Aisling	0 06	1 3	n.s.
Blenheim-Digger	0 40	23 2	**
Aisling-Digger	0 34	10 9	**
Aisling-Chad	0 39	8 0	**
Digger-Chad	0 05	0 05	n.s

* = difference significant at the 5% level; ** = difference significance at the 1% level; n s. = no significant difference

Table 7: Differences between ß-glucan levels due to varietal influences - 1991 barley samples

Variety vs Variety	Difference	F-value	Level of significance
Teal-Alexis	0 16	5 6	*
Teal-Grit	0 17	7 7	*
Alexis-Grit	0 01	0 0	n s
Alexis-Blenheim	0 13	3 5	n s
Grit-Blenheim	0 12	4 3	*
Grit-Aisling	0 31	37 2	**
Blenheim-Aisling	0 19	6.6	*
Blenheim-Chad	0 28	6 9	*
Aisling-Chad	0.09	0.81	n.s.

* = difference significant at the 5% level, ** = difference significant at the 1% level, n s = no significant difference

Table 8: Effect of season on ß-glucan levels on commonly grown varieties

	ß-glucan levels of barley grown in 1990	ß-glucan levels of barley grown in 1991	Δ
Grit	2 60	1.76	0.84
Blenheim	2 96** [1]	1 88*	1 08
Aisling	3 02 n s	2 07*	0.95
Chad	3 41**	2 16 n s.	1.25
Range	0 81	0.40	

[1]Statistical significance of the difference between two consecutive values
* = difference significant at the 5% level; ** = difference significant at the 1% level;
n.s = no significant difference

Table 9: Differences between ß-glucan levels, feed and malting barley samples

Oak Park			
Applied nitrogen	**Low F-value significance**	**Medium F-value significance**	**High F-value significance**
Pastoral - Puffin	n s	n.s.	n s
Pastoral - Blenheim	n s	*	*
Puffin - Blenheim	n s	*	*
Kildalton			
Applied nitrogen	**Low F-value significance**	**Medium F-value significance**	**High F-value significance**
Pastoral - Puffin	n s	*	*
Pastoral - Blenheim	n s	n s	n s.
Puffin - Blenheim	n s	*	*
Gusserane			
Applied nitrogen	**Low F-value significance**	**Medium F-value significance**	**High F-value significance**
Pastoral - Puffin	n s	n s	n.s.
Pastoral - Blenheim	n s	n s.	n s.
Puffin - Blenheim	n s.	n.s.	n.s.

* = difference significant at the 5% level, ** = difference significant at the 1% level,
n s = no significant difference

could be that crops sown later were subject to more drought stress during grain filling when ß-glucans are formed, a condition which did not arise in Ireland to the same extent.

2.3.2c Influence of applied nitrogen

Two sets of agronomic trials were carried out to evaluate the effects of applied nitrogen on barley ß-glucan levels. The first trial was the same as one of the trials used for the evaluation of varietal effects, three varieties were sown at Oak Park, Kildalton and Gusserane in three sets of six randomised plots in the autumn of 1991, and each set received high, normal or low rates of applied nitrogen at about the same time. In the second trial only one variety, Blenheim, was sown in the spring at the same three sites, low, medium and high rates of nitrogen were applied, but the timing of application was different at each site

ß-glucan levels obtained of each treatment of the above trials are shown in Tables 12 and 14 The listed values represent an average of six plot ß-glucan levels for each treatment. The coefficients of variation of the tabulated values are, with a few exceptions, about the same as that of the assay, thus indicating good reproducibility between plots

The difference between the ß-glucan levels of the Autumn sown barleys obtained at different rates of applied nitrogen is small, and the listed values do not show any general trend (Table 13) Small increases are noted in six, four and seven out of nine trials when rates of applied nitrogen are increased from low to medium, medium to high and low to high, respectively Only two increases - out of the possible twenty seven differences are statistically significant In Spring sown barleys, out of nine possible differences seven showed decreased ß-glucan levels with increased rates of applied nitrogen (Table 15), and only two of these are statistically significant. Altogether out of thirty six possible differences (Tables 13 and 15) between ß-glucan levels, due to increased rate of applied nitrogen in the two trials, sixteen showed decreased and nineteen increased levels, and only four of all the differences were statistically significant Since there is no general trend in the ß-glucan levels of the two trials due to changes in the rate of applied nitrogen, and only 11% of the total possible differences are statistically significant it is possible to conclude that the rate of applied nitrogen has no visible effect on β-glucan levels of barley grown here.

Table 10: ß-glucan levels at different sowing dates

	Oak Park			Kildalton		
	1	2	3	1	2	3
Date of sowing	26 Sept	16 Oct	5 Nov	15 Oct	6 Nov	21 Nov
ß-glucan level n = 6	2 09	1 99	1.93	2.26	2.32	2.12
s.d.	0.44	0 36	0 24	0 19	0 23	0.17
CV %	21 1	18 1	12.4	8.4	9 9	8.0

Table 11: Differences between ß-glucan levels at different sowing dates

Oak Park			Kildalton		
Sowing dates	**Difference between ß-glucan levels**	**F-value**	**Sowing dates**	**Difference between ß-glucan levels**	**F-value**
1-2	0 10	n s.	1-2	-0 06	n.s.
1-3	0 16	n s.	1-3	0.14	n.s.
2-3	0 06	n s	2-3	0.2	n s.

Table 12: Influence of rates of applied nitrogen of ß-glucan levels

	Oak Park			Kildalton			Gusserane		
Variety Blenheim									
Applied N	**Low**	**Medium**	**High**	**Low**	**Medium**	**High**	**Low**	**Medium**	**High**
ß-glucan level n=6	1 69	2 17	2 00	2 10	2 22	2 22	1 58	1 50	1 28
s d	0 33	0 15	0 15	0 57	0 08	0 15	0 18	0 41	0 13
CV %	19 5	6 9	7 5	27	3 6	6 8	11 4	27	10 2
Variety Pastoral									
ß-glucan level	1 51	1 70	1 56	2 09	2 15	2 17	1 34	1 48	1 38
s d	0 37	0 33	0 39	0 08	0 13	0 14	0 25	0 15	0 19
CV %	11 9	10 9	11 5	3 82	6 0	6 5	18 7	10 1	13 8
Variety Puffin									
ß-glucan level	1 41	1 54	1 66	1 86	1 74	1 85	1 40	1 30	1 40
s d	0 21	0 45	0 12	0 25	0 28	0.14	0 33	0 15	0.15
CV %	14 5	29 0	7 2	13 4	16 1	7 5	19 4	6 9	7 5

N = rate of applied nitrogen

Table 13: Differences between ß-glucan levels - applied nitrogen

Difference in ß-glucan level	Oak Park			Kildalton			Gusserane		
	Blenheim	Pastoral	Puffin	Blenheim	Pastoral	Puffin	Blenheim	Pastoral	Puffin
Medium-low	0 48*	0 19 n s	0 13 n s	0 12 n s	0 06 n s	-0 12 n s	-0 08 n s	0 14 n s	-0 10 n s
High-Medium	-0 17 n s	-0 14 n s	0 12 n s	0 0	0 02 n s	0 11 n s	-0 22 n s	-0 10 n s	0 10 n s
High-Low	31*	-0 05 n s	0 25 n s	0 12 n s	0 08 n s	-0 01 n s	-0 30 n s	0 04 n s	0 00 n s

Table 14: Influence of rates of applied nitrogen of ß-glucan levels

Variety Blenheim	Oak Park			Kildalton			Gusserane		
Applied N	Low	Medium	High	Low	Medium	High	Low	Medium	High
ß-glucan level	2 51	2 22	2 32	2 51	2 52	2 42	1 55	1 44	1 37
s d	0 4	0 17	0 16	0 27	0 35	0 26	0 15	0 13	0 08
CV %	15 9	7 7	6 7	6 7	13 9	10 7	9.7	9.0	5 8

Table 15: Differences between ß-glucan levels - applied nitrogen at three experimental sites

Var. Blenheim only	Oak Park	Kildalton	Gusserane
	Blenheim	Blenheim	Blenheim
Difference in ß-glucan level			
Medium - Low	-0 29*	0 01 n s.	-0.11 n.s.
High - Medium	0 10 n.s.	-0.10 n s.	-0.07 n s.
High - Low	-0.19 n.s.	-0.09 n.s.	-0 18*

* = difference significant at the 5% level n s. = no significant difference.

2.3.2d Environmental effects

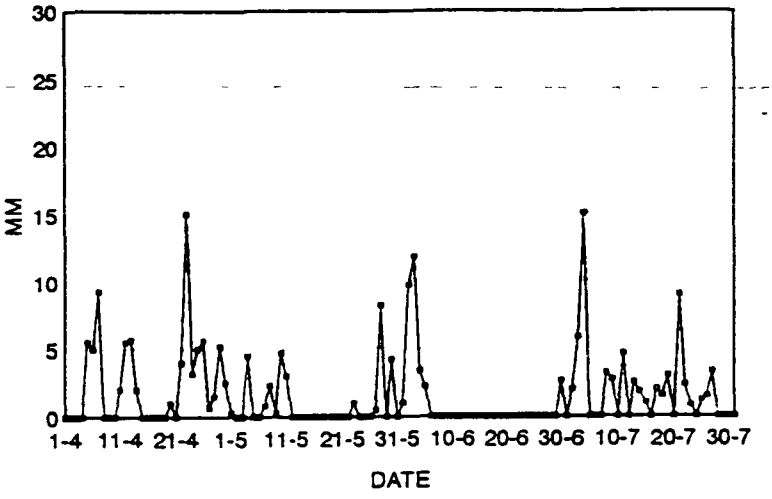
The trials for the determination of the effect of applied nitrogen on ß-glucan discussed in the preceding section could also be used to evaluate environmental influences Soils and climate in the three locations where the trials were carried out were sufficiently different to allow the study of environmental effects on ß-glucan levels

Temperature differences between the three locations were minimal and not likely to have significant effect on the growth of barley Average maximum daily temperatures during the four months of the barley growing season, namely April, May, June and July, differ only by 1 1°C

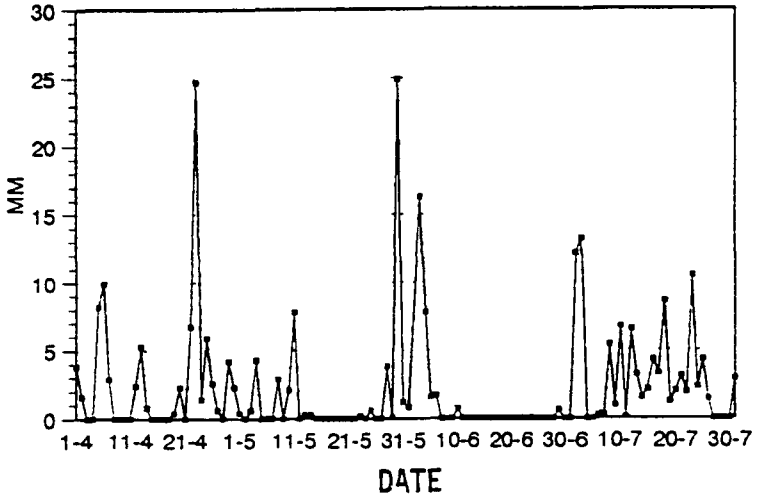
The largest difference between monthly averages is 1.3°C (Table 18) and the maximum difference between daily temperatures is 2°C, which occurred only three times and not on consecutive days

There are significant differences, however, between both the amounts and the pattern of rainfall in the three locations Total rainfall during the four months of interest was highest at Kildalton, followed by Gusserane and Oak Park (Table 19) Rainfall patterns were the same up to the end of May, and each location had a period of 25 to 27 consecutive days without rain in June (Figure 1). However, during the five days prior to the onset of the dry spell, the crop at Kildalton received twice as much rain (53 mm) as Oak Park (22 mm) or Gusserane (28 mm). Furthermore, after the dry spell during July 30% more rain fell in Kildalton than in Gusserane, and 80% more than in Oak Park.

RAINFALL DATA 1992
OAKPARK



RAINFALL DATA 1992
GUSSERANE



RAINFALL DATA 1992
KILDALTON

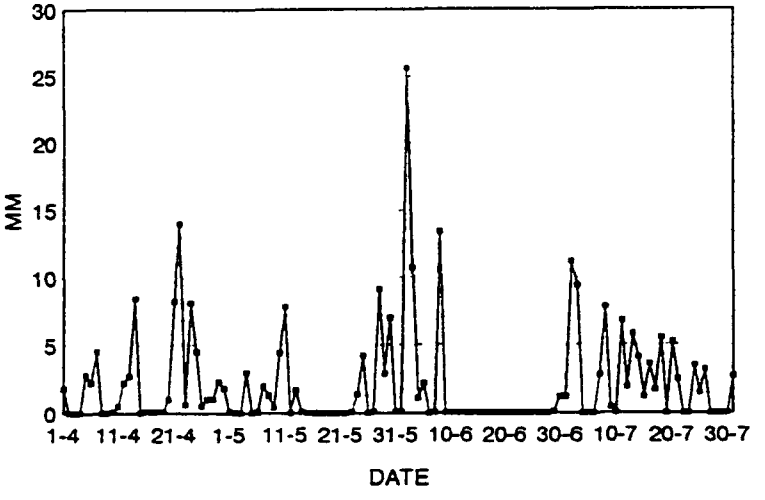


Figure 1

Apart from the differences in rainfall there are also some differences in the properties of soils. Soil composition is similar in the three locations. They are all grey podzols with nearly the same amounts of sand, silt and clay. The clay content of the soil at the Oak Park site is somewhat higher than that of the other two. Hence, it has a slightly better water holding capacity Soil textures at Oak Park and Kildalton are about the same, but the Gusserane soil is much lighter and loses water faster during a dry period than those at Oak Park and Kildalton.

Levels of ß-glucan obtained in the three environments for the varieties Blenheim, Pastoral and Puffin at low, normal and high levels of nitrogen are shown in Table 16. Precision associated with the listed values was discussed already under applied nitrogen (2.4 2c) According to results presented here highest ß-glucan levels are obtained at Kildalton and the lowest at Gusserane, in each case irrespective of variety used or applied nitrogen levels.

The largest differences in ß-glucan levels are those between Kildalton and Gusserane, ranging from 0.44% to 0 86% which correspond to 30 to 60% of the Gusserane values (Table 17) These values are highly significant - four at the 95% and five at the 99% confidence intervals Differences between Kildalton and Oak Park, and Oak Park and Gusserane ß-glucan levels are smaller, but still six and three values, respectively, are significant at the 95% confidence interval Furthermore, the trend in values is constant, Oak Park ß-glucan levels are lower than those of Kildalton, and Gusserane levels are lower than those of Oak Park in each case It can be concluded, therefore, with reasonable certainty that between the three locations, environmental factors prevailing at Kildalton lead to the highest, and those in Gusserane to the lowest ß-glucan levels

In order to simplify the interpretation of environmental effects on ß-glucan levels, climatic differences were considered only during the formation of ß-glucans It is assumed that ß-glucans in barley plants are formed at the same time as grain starch, which takes place within about 35 days following ear emergence. Winter barley ear emergence in 1992 occurred around the last week of May, hence the influence of environmental factors on ß-glucan formation would be most significant in June

All three locations had a long dry period in June, but during the five days before the onset of the dry spell twice as much rain fell at Kildalton than at the other two sites. Considering that the soil at Kildalton has the same water holding capacity as that at Oak

Park, and higher than at Gusserane, it must have had the highest soil moisture during the rain-free period. As both Oak Park and Gusserane had the same rainfall before the dry spell, the former because of its higher water holding capacity should have a higher soil moisture level during the period of importance Consequently relative soil moisture levels in June should be in the order of Kildalton > Oak Park > Gusserane.

Differences in soil moisture levels during the dry period seem to be reflected in ß-glucan levels. Barley grown at Kildalton where soil moisture is expected to be the highest has also the highest ß-glucan level, and similarly soil with the lowest moisture, that is the Gusserane sites, yields the crop with the lowest ß-glucan level. It can be concluded, therefore, that ß-glucan level in barley is directly proportional to soil moisture during grain filling. The conclusion reached here, however, is based only on observations in a year when the period of grain filling coincides with water deficiency, and it will not necessarily apply to growing seasons when soil moisture is not a limiting factor during the same period

Our observation on environmental effects on β-glucan levels is at variance with Canadian studies (Bendelow, 1975) where it was found that β-glucan levels are inversely proportional to moisture in the growing environment These contradictory findings can be explained in terms of rates of grain filling In the Irish

Table 16: Average monthly temperatures in °C, spring 1992

	April	May	June	July	Average
Oak Park	12 5	17 6	20 4	19 9	17 6
Kildalton	12 3	17 4	19 8	19 2	17 2
Gusserane	11 5	16 3	19 6	18.7	16 5

Table 18: ß-glucan levels as determined at three experimental sites

Variety	Blenheim			Pastoral			Puffin		
Location	**OP**	**KD**	**GUS**	**OP**	**KD**	**GUS**	**OP**	**KD**	**GUS**
Low N									
ß-glucan levels n = 6	1 69	2 10	1 58	1 51	2 09	1.34	1.41	1 86	1.40
s d	0 33	0 57	0 18	0 37	0 08	0 25	0 21	0.25	0.33
CV %	19 5	27 0	11 4	24 5	3 82	18 7	14 5	13 4	19.4
Normal N									
ß-glucan level n = 6	2 17	2 22	1 50	1 70	2 15	1 48	1 54	1.74	1 30
s d	0 15	0 08	0 41	0 33	0 13	0 15	0.45	0 28	0.15
CV %	6 9	3 6	27 0	19 4	6 0	10 1	29 0	16 1	6 9
High N									
ß-glucan level n = 6	1 00	2 22	1 28	1 56	2 17	1 38	1 66	1 85	1 40
s d	0 15	0 15	0 13	0 39	0 14	0 19	0 12	0 14	0.15
CV %	7 5	6 8	10 2	25	6 6	13 8	7.2	7.5	7.5

OP = Oak Park, KD = Kildalton, GUS = Gusserane;
N = rate of applied nitrogen

Table 19: Differences between ß-glucan levels - environmental influences

Difference in ß-glucan levels	Low N			Normal N			High N		
	Blenheim	**Pastoral**	**Puffin**	**Blenheim**	**Pastoral**	**Puffin**	**Blenheim**	**Pastoral**	**Puffin**
OP - KD	-0 41*	-0 58*	-0 45*	-0 05 n.s.	-0.45*	-0.20 n.s.	-0.22*	-0 61*	-0.19 n.s.
OP - GUS	0 10 n s.	0 17 n s	0 01 n s	0 67*	0 22 n s	0 24 n s.	0 62*	0.18 n.s	0.26*
KD - GUS	0.52*	0 85**	0 46*	0.72**	0 67*	0 44*	0.84**	0 79**	0.45*

OP = Oak Park, **KD** = Kildalton; **GUS** = Gusserane
* = difference significant at the 5% level, ** = difference significant at the 1% level, n s = no significance

Table 17: Monthly total rainfall in mm, spring 1992

	April	May	June	July	Total
Oak Park	78 7	30.0	30 7	71 8	211
Kildalton	58 6	45 6	54 5	128 8	287
Gusserane	64 6	31 4	29.9	98 8	225

environment grain filling took place during a period of restricted growth due to water deficiency, and the crop with the highest soil moisture had the highest rate of grain filling and also the highest ß-glucan level. It is possible, therefore, that the final ß-glucan level in the grain is directly proportional to the rate of grain filling. In the North American environment drier areas could imply higher all round temperatures, and crops grown in these locations could mature faster and, thus, have a higher rate of grain filling than those grown in a wetter and cooler environment Unfortunately no weather data was included in the Canadian study to confirm our hypothesis

2.5 CONCLUSIONS

The comparative study of the lichenase (McCleary and Codd, 1981) and the cellulase (Carr *et al* , 1991) assays was not entirely conclusive It can be argued that it is more advisable to use the lichenase assay because it is accepted by the European Brewery Commission and there is some, although not necessarily indisputable, evidence to indicate that the assay does determine total β-glucan levels in barley On the other hand it could also be argued that cellulase assay gives a more accurate measure of β-glucan levels than the lichenase assay, because the obtained values are considerably higher and at present there is no experimental evidence to indicate that the determined glucose originates from a source other than β-glucan

There is a definite varietal effect on the β-glucan levels of Irish malting barley varieties studied here and it could be observed for two successive years Similarly environmental influence on the β-glucan levels of three barley varieties, Blenheim, Pastoral and Puffin have been observed. β-glucan levels seem to decline as soil moisture levels decrease during the period of grain filling

Agronomic factors had no obvious effect on the β-glucan levels of the standard varieties Differences in Autumn sowing dates caused no visible changes in β-glucan

levels, and differences in the rates of applied nitrogen had no clear cut effect on the same. While some differences in β-glucan levels between two rates of applied nitrogen were significant the data was not conclusive enough to indicate a trend of β-glucan levels with changing rates of applied nitrogen.

CHAPTER 3

AGRONOMIC AND ENVIRONMENTAL EFFECTS ON ß-GLUCANASE LEVELS IN IRISH BARLEY

3.1 INTRODUCTION

3.1.1 β-glucanases in malted barley.

Most β-glucans in barley are found in the cell walls of the starchy endosperm (Fincher, 1975). During germination the starchy endosperm becomes mealy and friable which is mainly due to the dissolution of the cell walls. The process allows the food reserves in the endosperm to be exposed to enzymes released during germination, which will hydrolyse them. However, not only the starchy food reserves are hydrolysed, but also the levels of ß-glucan and newer cellulose declined significantly, and after ten days of germination only 25% of the former remained in the barley (Bamforth, 1982). Consequently there must be an enzyme or a group of enzymes which are responsible for the digestion of β-glucans

Several workers have observed the dissolution of barley cell walls in malt or barley flour extracts (Scott, 1972 and references therein), but it was not until 1979 that an enzyme involved in the β-glucan degradation, was purified and characterised (Bamforth *et al*, 1979) The enzyme was termed solubilase, because it dissolved β-glucan in cell walls, and analysis indicated it to be a carboxypeptidase, which showed no activity on β-glucan extracted from barley Its main role is probably the hydrolysis of protein β-glucan linkages which hold the cell wall together Solubilase was shown to be very heat stable, and as there is usually an excess of it in malt (Bamforth and Martin, 1981) there is no value in specifying its activity

Three other enzymes have been identified later as having a possible role in the degradation of β-glucans and these are collectively known as endo β-glucanases Endo-β1-4 glucanase is mostly found in the husk where it is thought to degrade cellulose (Hoy *et al*, 1981) While small quantities of the enzyme are found in grain, most of it originates from micro-organisms which contaminate the husk. Endo-β1-3-glucanase is found mainly in the aleurone layer, it has no action on β-glucan from flaked barley, but reduces the viscosity of green malt extract (Bathgate *et al.*, 1974). It is possible that the enzyme hydrolyses β-glucans which are dissolved in the early stages of malting Neither of these two enzymes have been purified and their exact role in the digestion of β-glucans is not known

The third endo-β-glucanase, endo-barley β-glucanase is probably the only enzyme in the group which is known to have a definite role in the digestion of β-glucans. The

enzyme has been obtained from germinating barley in purified form, and it was shown to hydrolyse specifically the β1-4 bonds of the β(1-3) (1-4) bonded glucose units of the β-glucan chain (Woodward and Fincher, 1983). The action of endo-barley ß-glucanase on ß-glucan is the same as that of other β-glucanases obtained from fungal and bacterial sources, namely laminarinase (Parrish *et al,* 1960) and lichenase (McCleary and Glennie-Holmes, 1985). In each case β-glucan is hydrolysed to a mixture of mainly cellotriosyl and cellotetrasyl units. (p.3 Chapter 2).

In contrast to "solubilase" only a very small amount of endo-barley β-glucanase is found in barley, which however increases rapidly upon germination (Bourne and Pierce, 1970). The enzyme is not heat stable and about 50% is inactivated during kilning (Bourne and Pierce, 1970). Drying at low temperature initially (approx. 38°C) before increasing the heat supply leads to greater survival rate of the enzyme (Bamforth, 1982).

Because endo barley β-glucanase is the only known enzyme in malt to depolymerise barley β-glucan it is an important quality parameter. The importance of β-glucanase in brewing and nutrition, and analytical methods for the determination of the same, will be discussed in the subsequent sections.

3.1.2 Importance of β-glucanase in brewing

In most breweries an adjunct normally milled or flaked barley is used along with malted barley as a source of fermentable carbohydrates. During extraction of carbohydrates from the grain, referred to as mashing, cell walls of the barley endosperm are dissolved by the " solubilase" from the malted barley, and the stored starches become accessible to hydrolysis by amylolytic enzymes. Along with the solubilisation starches the enzyme "solubilase" also releases β-glucans from the endosperm cell walls. β-glucans increase the viscosity of the extract referred to as wort, and adverse effects of increased wort viscosity on the brewing process was discussed before (section 2.1.1.).

It should be possible to reduce the viscosity of the wort by depolymerising β-glucans with β-glucanase either from the malted barley or from external sources. According to a study carried out by Bourne and Pierce (1970), malt with high β-glucanase activity reduced the viscosity of the wort considerably more than malt with low β-glucanase activity. Addition of extra barley-ß-glucanase extracted from malt to the wort reduced the viscosity even further than malt alone. The results indicate conclusively that the viscosity of wort is effected by the β-glucanase activity of the malt used. Incomplete

degradation of β-glucan therefore suggests that there is insufficient β-glucanase present even in mash made with high β-glucanase activity malt - probably because much of the enzyme is inactivated at mashing temperatures of 65°C

β-glucanases from microbial sources are much more heat stable than barley β-glucanases Crabb and Bathgate (1973), found that the addition of small amounts (0 05% (w/w) on grist fungal β-glucanase to the mash at 65°C reduced wort viscosity even further than malt with very high β-glucanase activity However the high cost of microbial β-glucanase mitigates against the widespread use of the enzyme in brewing.

A study on the heat stability of barley β-glucanase indicated that enzyme activity declined fast at 65°C, but about 35% of the enzyme remained at 50°C after 20 min (McCleary, 1986) The author suggested that m order to make maximum use of the β-glucanase present in malt, an initial mashing stage at 50°C should be incorporated into the brewing process

It is evident from the foregoing discussion that β-glucanase plays a significant role in the brewing process Consequently the level of β-glucanase is an important quality parameter of malted barley Methods for the determination of β-glucanase in malt will be discussed in the next section

3.1.3 Survey of analytical methods for the determination of β-glucanase

There is a considerable interest in a rapid and reliable method to determine ß-glucanase levels in malts due to the role of these enzymes in the depolymerisation of ß-glucan during malting and most probably mashing Three procedures have been developed and incorporated in the recommended methods of the Institute of Brewing Collaborative studies have been carried out in order to compare ß-glucanase determinations in malted barley by these three techniques The three extensively used techniques are viscometry (Bourne and Pierce, 1970) radial gel diffusion (Martin and Bamforth, 1983) and dye labelled substitute assays (McCleary and Shameer, 1987)

Viscometric technique involved in the determination of ß-glucanase activity to measure the reduction in the kinematic viscosity of a digest containing a malt extract and ß-glucan substrate Bathgate, (1979) concluded that ß-glucanase levels can be accurately assayed by the viscometric procedure of Bourne and Pierce (1970) provided a common standard β-glucan substrate is used by each laboratory Also the results of each series of determinations should be corrected against the current Institute of Brewing check malt.

In 1981 a paper on the use of dye-polysaccharide interactions in ß-glucanase assay was published (Wood, 1981). The report indicated that radial diffusion of ß-glucanase into a dye ß-glucan complex bearing gel slab to be an efficient and inexpensive method for ß-glucanase determination. The area of reaction was proportional to the logarithm of the amount of enzyme and could be visualised by a variety of methods such as clearing of opaque substrate and various staining.

Following this report a radial diffusion assay was developed specifically for the quantification of endo ß-glucanase activity (Martin and Bamforth, 1983). The method involves an assay where a Congo Red-β-glucan complex in agarose gel is digested by the diffusion of ß-glucanases across the gel, hence releasing the dye (bound to ß-glucan) and thereby producing a clear zone. Congo Red does not interact with the oligosaccharides which are produced from ß-glucan by digestion with ß-glucanase. The enzyme diffuses into the gel and the amount of enzyme is directly proportional to the diameter of the clearing zone. The technique is simple and less time consuming than viscometry. Plates can be prepared in bulk and stored at 4°C prior to use, obviating the need for solutions of ß-glucan required in the viscometric technique.

In addition to the total radial diffusion assay a colorimetric method for the determination of ß(1-3) glucanase was also developed. The enzyme substrate was Cibracon Blue F3GA complexed to a dextrinase-treated streptococcal glucan. This assay proved to be very efficient in the screening of large batches of samples and could be adapted to quantitative and qualitative application in relation to ∝(1-3) glucanases produced from bacterial sources (Lamberts *et al.*, 1981).

The above technique provided the impetus to devise a more reliable and efficient procedure for ß-glucanase determinations (McCleary 1986; McCleary adn Shameer, 1987) in malt. The new assay involved the incubation of buffer extracted ß-glucanases (present in malt supernatants) and a chemically-modified ß-glucan substrate complexed to Remazol Brilliant Blue dye at 30°C. ß-glucanase in the malt extract released the blue dye from the complex into solution and the colour change could be quantified by spectrophotometry.

The amount of dye released is directly proportion to the amount of ß-glucanase and a calibration curve can be drawn using known ß-glucanase activities which can be used to determine the ß-glucanase activity in unknown malt samples. The technique proved to be very precise and suitable for large numbers of assays and it was incorporated into a kit which is available commercially.

The Cereal Chemistry Division of the Royal Australian Chemical Institute had carried out extensive collaborative tests (Buch, 1986) on the dye labelled ß-glucanase (McCleary and Shameer, 1987) and radial diffusion assay (Martin and Bamforth, 1983) and viscometry (Bourne and Pierce, 1970). The study involved the analysis of six samples by eighteen Australian laboratories and three European laboratories by the three methods already described The dye labelled ß-glucanase assay showed both the best intra- and inter-laboratory reproducibilities r=8.4% and R=16.7%, followed by radial diffusion assay, r=19.7% and R=33 7% The poorest reproducibility was obtained by viscometry, r=19.1 % and R=58.0%. The symbol "r" represents average coefficient of variation of intra-laboratory determinations, and "R" the coefficient of variations of the average values obtained by the participating laboratories. It was concluded that the azo-barley substrate method proved to be superior in precision and reproducibility, and it would be worthwhile to have the assay adopted by malsters and brewers as a new standard method

3.1.4 Objectives

Most work on β-glucanase concentrated on the analytical methods, but to the best of our knowledge no report has been published so far on varietal and external effects on levels of the same on malt The objective of this part of our work is to evaluate the influence of varietal and environmental factors on the β-glucanase levels in malt Strictly speaking it is not the effect on β-glucanase which is being evaluated, as it is not present in appreciable quantities in barley, but rather the effect on the precursors which are involved in the synthesis or release of the enzyme

In order to carry out the above study it will also be necessary to evaluate the precision of the analytical method used and determine if it is suitable for the rapid analysis of the large number (200+) of samples necessary for the present work Furthermore as the samples used for the determination of average β-glucanase level for each treatment or variety are micro-malted separately, the effect of the process on the precision of the obtained values will also be evaluated.

3.2 MATERIALS AND METHODS

3.2.1 Determination of β-glucanase using Azo barley β-glucan substrate technique

3.2.1a Materials

Barley samples, all certified varieties, were supplied by the Department of Agriculture, Forestry and Food or the breeder, and were micro-malted at the Guinness and Son Ltd., Laboratories, Dublin The β-glucanase determination kit was supplied by Megazyme Pty Ltd., Sydney, Australia.

3.2.1b Preparation of reagents and substrates.

All reagents were of analytical grade, except for methyl cellosolve which was laboratory grade

Azo barley glucan substrate Chemically modified, dye-labelled barley beta-glucan (1% w/v) sodium azide was supplied in a β-glucanase assay kit by Megazyme Pty Ltd. (Australia) It was stored at 0 4°C between use

Concentrated buffer 800 M sodium acetate plus 800 M sodium phosphate buffer stabilised in 0 02% sodium azide was prepared 25 mls of the concentrate was diluted to 500 ml with distilled water before use It was stored at 4°C between use

Precipitant solution 40 0 g sodium acetate and 4 0 g zinc acetate was dissolved in 150 ml distilled water The pH was adjusted to 5 0 with concentrated hydrochloric acid and volume adjusted to 200 ml To this solution 300 0 ml of methyl cellosolve was added and mixed well It was stored at room temperature

Extractant buffer solution 5 44 g of sodium acetate and 6 24 g of sodium dihydrogen orthophosphate was dissolved in 900 ml of distilled water, and 0 2 g of sodium azide was added The pH was adjusted to 4 6 by the addition of 1 M hydrochloric acid and volume was adjusted to 1 0 litre This was stored at 0-5°C

3.2.1c Methodology of ß-glucanase determination

Malt samples were milled to pass 0 5 mm screen and about 0 5 g was accurately weighed into 12 ml capacity pyrex test tubes. Extractant buffer (8 ml) was added to each tube and the contents were occasionally swirled over a one-hour period at room temperature and were centrifuged (1000 g) for 10 minutes

A 0 5 ml sample of the malt extract which had been pre-incubated at 30°C for 5 minutes was added to the test tubes containing 0.5 ml azo-barley beta-glucan substrate,

also at 30°C. The test tubes were vigorously stirred on a vortex and incubated at 30°C for exactly 10 minutes

0 5 ml azo-barley beta-glucan substrate was dispensed into each test tube and incubated for 10 minutes at 30°C. The reaction was terminated by the addition of 3 ml of precipitant solution and tube contents were mixed vigorously or vortexed. After standing at room temperature for 5 minutes the tubes were centrifuged (1000 g) for 10 minutes and absorbance of the clear supernatant solution read against a reaction blank at 590 nm. The reaction blank was prepared by adding 3 ml of precipitant solution to 0.5 ml of azo-barley beta-glucan before the addition of crude malt extract

3.2.1d Calculation of ß-glucanase levels

Malt ß-glucanase is calculated using the equation

$$Y = MX + C$$

where

Y = malt ß-glucanase activity (in U/kg of malt)

M = slope of calibration graph

X = absorbance of reaction at 590 nm (minus absorbance of blank)

C = intercept on y-axis

values for M and C are given by the supplier for every batch of azo-barley ß-glucan

3.2.2 Micro-malting procedure

All samples assayed for ß-glucanase levels were previously micro-malted under standard conditions in A Guinness and Son Ltd , Laboratories, St. James Gate, Dublin under the following conditions The initial stage of micro-malting involves steeping 6 hour wet, 4 hour air rest, followed by another 6 hour wet period, 4 hour air rest and 12 hour wet period at 16°C The germination period was 122 hours at 16°C followed by the final kilning stage where germinated barley grains were dried for 16 hours at 57°C and then followed by a 12 hour period at 75°C

3.2.3 Attempted determination of ß-glucanase by liquid chromatography

3.2.3a Extraction of ß-glucanase

The same method was used for the extraction of ß-glucanase from malt as in the dye labelled ß-glucan ß-glucanase assay (Section 3 2.1c)

3.2.3b Apparatus

The chromatofocusing column, Mono P 200 x 5 mm, used for the separation of malt proteins, was connected to a Pharmacia FPLC* System (both from Pharmacia Biosystems Ltd., Milton Keynes, U K.)

3.2.3c Chromatographic conditions

The following buffers were used to obtain an elution pH range of 7-5 Equilibrium buffer, 0 025 M bis Tris (Tris-2-amino-2-(hydroxymethylpropane-1,3-diol) adjusted to 7 1 with 2 M hydrochloric acid, eluent buffer, 10 ml Polybuffer* 74 diluted to 95 ml with water, followed by pH adjustment to 4.0 with 2 M hydrochloric acid and dilution to 100 ml.

The sample was injected after stabilising column pH with equilibrium buffer and passing 3 ml eluent buffer through it The sample was eluted with a further 43 ml eluent buffer The column was stabilised with the equilibrium buffer after elution Typical injection volumes were 100 μl and the flow rate was 0 5 ml/min. Procedures for column cleaning were described in section 1 2 5a

*Polybuffer and FPLC are trade names of Pharmacia Biosystems Ltd

3.3 RESULTS AND DISCUSSION

3.3.1 Evaluation of the analytical method

According to a repeatability study carried out by the Royal Australian Chemical Society, on the reported ß-glucanase assays namely, viscometry, radial diffusion and dye labelled ß-glucan assays, the dye labelled assay gave the best intra and inter-laboratory reproducibilities (Buch, 1986) The assay is also the simplest of the three, it is less subject to operator errors, the required dye-ß-glucan substrate along with the buffers and controls can be obtained commercially, and it is much faster than the other two methods Consequently it was decided that the dye-labelled ß-glucan assay is the most suitable for the evaluation of the influence of external factors on ß-glucanase levels in malted barley

The repeatability of the dye labelled ß-glucan assay in our laboratory was evaluated by carrying out six repeat determinations on six Irish malted barley varieties, and on an unspecified Australian malt variety which was supplied as control.ß-glucanase level of the control sample was found to be within 0.1 U/kg of the reported value,(Table 1) and the repeatability was considerably better than reported by the previous study (3 2

vs 8 4%) (Buch, 1986). For the Irish malted barley varieties the obtained repeatability was poorer, it varied from 6 to 14%, and was different for each variety However, the average repeatability was not very different from that reported for Australian malted barleys (8 4 vs 10%).

Apart from the repeatability of the assay the effect of separate micro-malting on the precision of the ß-glucanase level was also evaluated. As ß-glucanase levels in the present study were determined by averaging six ß-glucan levels of separately micro-malted samples it is necessary to know whether the variability of micro-malting effects the precision of the final ß-glucanase value

Barley samples from the Teagasc six variety trials, six samples for each variety from six different plots, were used to determine the variability of micro-malting These samples were uniform because the variation of the ß-glucan levels in each set of six samples (3 5-9 7%) (Table 4, Chapter 2), was no larger than the variation of the ß-glucan assay (4-10%) (Table 2, Chapter 2)

The six samples of each variety were micro-malted separately and obtained average ß-glucanase levels for each variety along with corresponding coefficients of variation are shown in Table 2. The variation of the average ß-glucanase level of each variety is much larger in four out of six varieties when separately micro-malted samples were used than when repeat determinations were carried out on the same sample The coefficient of variation of repeat determination ranges from 4 to 14% whereas those of the separately micro-malted sample from 10 to 38%, hence separate micro-malting does add an extra variation to the determined ß-glucanase level

Poor heat stability is probably the reason for the variation of ß-glucanase levels with micro-malting One of the stages in the micro-malting process involved the drying (kilning) of germinated barley between 50 and 60°C It has been reported that about 50% of the ß-glucanase degrades during kilning, (Bourne and Pierce, 1970) and enzyme denaturation is not necessarily uniform from sample to sample

3.3.2 External effects on ß-glucanase levels in malted barley

3.3.2a Varietal effect

To evaluate varietal effect on ß-glucanase in malted barley the samples used to study the same effect on ß-glucan in barley were micro-malted.ß-glucanase levels found in malted barleys corresponded to barley varieties from the 1990 and 1991 variety trials

Table 1: Varietal influence of ß-glucanase levels of barley grown in 1990

Variety	Blenheim	Chad	Chef	Digger	Aisling	Grit	Control (malt)
ß-glucanase level (U/kg) n = 6	580 2	407 4	481 4	215 5	269 2	362 0	294.6
s.d.	53 9	57 5	49 7	25 9	16 2	26 0	11 7
CV %	9 3	14	10 3	12	6	8	3 9

Table 1a: Differences in ß-glucanase levels of barley grown in 1990

Variety vs Variety	Difference in ß-glucanase levels	F-value	Level of significance
Blenheim vs Chad	172 83	28 84	*
Blenheim vs Chef	98 86	10 92	*
Blenheim vs Digger	364 78	223 23	**
Blenheim vs Aisling	310 99	183 14	**
Blenheim vs Grit	254 21	108 10	**
Chad vs Chef	-73 97	5 69	*
Chad vs Digger	191 92	55 56	**
Chad vs Aisling	138 16	32 07	*
Chad vs Grit	81 38	9 97	*
Chef vs Digger	265 92	135 32	**
Chef vs Aisling	212 13	99 0	*
Chef vs Grit	155 55	46 07	*
Digger vs Aisling	-53 79	18.62	*
Digger vs Grit	-110 57	54 43	**
Aisling vs Grit	-56 78	20 59	*

* = significant at 5%, ** = significant at 1%, n s = not significant

Table 2: Varietal influence on ß-glucanase levels of barley grown in 1990

Variety	Blenheim	Grit	Alexis	Teal	Chad	Aisling
ß-glucanase level (U/kg) n = 6	323 79	252 56	324 68	269 18	263 07	224 61
s.d.	72 12	35 35	81 73	50 01	48 85	21 86
CV %	22 3	14 0	25 1	18 6	37 6	9.7

Table 2a: Differences between ß-glucanase levels of barley grown in 1991

Variety vs Variety	Difference	F-Level	Level of significance
Teal vs Alexis	-55 5	2 01	n s
Teal vs Grit	16 62	0 44	n s
Alexis vs Grit	72 12	3 94	n s.
Alexis vs Blenheim	0 89	0 00	n s
Grit vs Blenheim	-71 23	4 72	*
Grit vs Aisling	27 95	2 71	n s
Blenheim vs Aisling	99 18	10 39	*
Blenheim vs Chad	60 72	1 48	n s
Aisling vs Chad	-38 46	0 87	n s

* = significant at 5%, ** = significant at 1%, n s = not significant

are shown in Tables 1 and 2 The tabulated values of the Department of Agriculture trials each represent an average of six determinations on one bulked sample, composed of grain from different growing areas, whereas those of the Teagasc trial use an average of six values from separately micro-malted samples for which the barley was obtained from six different plots.

The CVs of the found ß-glucanase levels of malted barleys in the 1991 trial are considerably higher than those from the 1990 trials. It was explained in section 3 3 1 that the large coefficients of variations are due to the variability of micro-malting. The range of ß-glucanase levels 215-580 U/kg in malted barleys from the 1990 variety trials is evidence for strong varietal effect. The varietal effect is further confirmed by the significance of the differences between ß-glucanase levels of the eight malted barley samples (Table 1a) All the possible differences between the ß-glucanase levels of the eight varieties are statistically significant including those between consecutive values. Found varietal ß-glucanase levels increase in the order of Digger, Aisling, Grit, Chad, Chef and Blenheim

Varietal effects on ß-glucanase levels are not pronounced in the 1991 as in the 1990 variety trial The range of values is narrower (224-324 U/kg vs 215-580 U/kg) and differences between all varieties and also those common to the two trials are considerably smaller (Table 2a), but the relative order of values, Blenheim, Chad, Grit, Aisling is the same in the two trials Only two of all the possible differences between varieties are significant namely between Grit and Blenheim and Aislmg and Blenheim, whereas all differences were significant in the 1990 trial However, the fact that some of the differences are not significant in the six variety trials is probably due to the high coefficient of variation Considering that the smallest significant difference in the 1990 trial is 52 U/kg, differences between Teal and Alexis 56 U/kg, Alexis and Grit 72 U/kg, Blenheim and Chad 61 U/kg, in the six variety trials would be significant, if the coefficients of variations were low as in the former Thus, the results of the 1991 trial indicate that even if varietal ß-glucanase levels and inter-varietal differences vary from season to season, the varietal effect is still evident and the order of values remains constant

The purpose of the next set of trials was to examine if varietal effects remain constant with changing environmental and agronomic factors. Three barley varieties, namely Blenheim, Pastoral and Puffin, were grown at Oak Park, Kildalton and

Gusserane and representative samples were micro-malted.

In all three trials (Table 3), malting barley from variety Blenheim had the highest level of ß-glucanase, followed by the feed barleys Puffin and Pastoral. ß-glucanase levels of a single variety are different in each location. The differences between Blenheim and Pastoral (Table 3a) are the largest, 250-67 U/kg and seven out of nine differences are significant. The differences between Pastoral and Puffin are somewhat smaller 148-46 U/kg and six out of nine trials are significant. However in both sets of ß-glucanase levels - all the differences would be significant if the coefficients variation were of the same order as obtained for the 1990 trials. Differences between the ß-glucanase levels of malted barleys Blenheim and Puffin (110-4 U/kg) are smaller than between the other two varieties and only one out of the nine values are significant. However in each case Blenheim has higher ß-glucanase level than Puffin, and again we can assume that if the coefficient of variation of the assay could be improved most of the differences between the two varieties would be significant. Thus the data obtained from the above trial indicate that while ß-glucanase levels can vary with growing environment and agronomic practices, the inter-varietal order remains the same, i.e., Blenheim >Puffin >Pastoral >remains constant.

3.3.2b Effect of sowing date

In order to evaluate the effect of barley sowing date on the ß-glucanase levels, barley samples used for the evaluation of the same effect on ß-glucan were micro-malted. Particulars of the agronomic trials are described in section 2.2.3.

Obtained ß-glucanase levels are shown in Table 4 and the listed values represent an average value of six separately micro-malted samples. Coefficients of variation of five out of the six samples were in the same range as those obtained for repeat determinations of ß-glucanase levels of the same sample (Table 1). Hence the contribution of micro-malting to the error of the listed value of ß-glucanase must be minimal in this particular case.

Accordingly the listed values (Table 4) sowing date at Oak Park had no effect on the ß-glucanase levels of malted barley. The obtained ß-glucanase levels do not show an upward or downward trend, and the differences between the values are small and not significant (Table 4a). On the other hand ß-glucanase levels obtained for malts from barley grown at Kildalton show a small increase with each consecutive sowing

Table 3: Varietal influence on ß-glucanase levels in malting and feed barleys

	Applied nitrogen level	Location (1) Oak Park			Location (2) Kildalton			Location (3) Gusserane		
		Pastoral	Puffin	Blenheim	Pastoral	Puffin	Blenheim	Pastoral	Puffin	Blenheim
ß-glucanase levels (U/kg) n = 6	Low	237 91	330 87	397 39	376 71	422 27	492 94	319 69	467 69	540 46
s d		37 61	38 95	53 70	81 47	78 05	27 64	98 51	104 42	28 10
CV (%)		15 8	11 78	13 5	21 6	18 5	5 6	30 80	22 30	5 20
ß-glucanase level (U/kg) n = 6	Medium	331 21	415 37	444 52	331 21	415 37	444 52	231 83	458 48	482 22
s d		62 19	55 52	86 25	62 19	55 52	86 52	88 78	103 66	80 74
CV (%)		18 8	13 4	19 4	18 8	13 4	19 50	38 30	22 60	16 70
ß-glucanase level (U/kg) n = 6	High	250 87	332 94	442 81	380 87	444 07	448 14	269 05	393 95	486 05
s d		18 31	15 53	61 36	45 15	56 49	101 00	93 15	91 55	108 12
CV (%)		7 2	4 7	13 90	11 90	12 72	22 50	34 60	23 20	22 20

Table 3a: Differences between ß-glucanase levels, feed and malting barley varieties

Applied nitrogen	Low			Medium			High		
	Difference	Significance	F-Value	Difference	Significance	F-Value	Difference	Significance	F-Value
Oak Park									
Pastoral - Puffin	-92 95	*	17 69	-84 16	n s	0 29	-82 07	*	70 09
Pastoral - Blenheim	-159 48	*	35 51	-113 31	n s	0 88	-191 94	*	53 91
Puffin - Blenheim	-66 52	*	5 99	-29 15	n s	0 54	-109 87	*	18 08
Kildalton									
Pastoral - Puffin	-45 56	n s	0 98	-84 16	*	6 11	-63 20	n s	4 58
Pastoral - Blenheim	-116 23	*	10 95	-113 31	*	6 81	-67 27	n s	2 22
Puffin - Blenheim	-70 67	n s	4 37	-29 15	n s	0 48	-4 07	n s	0 01
Gusserane									
Pastoral - Puffin	-148 00	*	5 84	-226 65	*	13 80	-124 95	*	5 49
Pastoral - Blenheim	-220 77	*	27 87	-250 39	*	24 01	-217 00	*	13 87
Puffin - Blenheim	-72 77	n s	2 53	-23 74	n s	0 18	-92 10	n s	2 54

* = significant difference at the 5% level, n s = no significant difference

Table 4: Differences between ß-glucanase levels at different sowing dates

	Oak Park			Kildalton		
Date of sowing	1 26 September	2 16 October	3 5 November	1 15 October	2 6 November	3 21 November
ß-glucanase level (u/kg) n = 6	320 0	337 0	319 0	304 0	318 5	351 9
s d	31 7	29 7	50 8	39 9	30 9	18 56
CV (%)	9 9	8 8	15 9	13 0	9 7	5 3

Table 4a: Differences between ß-glucanase levels at different sowing dates

Oak Park			Kildalton		
Sowing dates	**Difference between ß-glucanase levels**	**F-Value**	**Sowing dates**	**Difference between ß-glucanase levels**	**F-Value**
1-2	-16 80 n s	0 90	1-2	-14 4 n s	0 49
1-3	1 01 n s	0 00	1-3	-47 8*	7 06
2-3	17 81 n s	0 55	2-3	-33 4*	5 13

* significant at the 5% level, n s = no significant difference

date and the differences between the second and the third sowing dates are significant. However, in the absence of supporting data it is difficult to attribute the observed difference to the later sowing date, because the increase is very small and the ß-glucanase levels obtained at Kildalton at a later sowing date are not significantly higher than those obtained for earlier sowing dates at Oak Park. Consequently it must be concluded that Autumn sowing dates in 1991, had no effect on the ß-glucanase levels of the malted barleys from the subsequent harvest.

3.3.2c Environmental effects

Environmental differences between the three locations, namely Oak Park, Kildalton and Gusserane have been discussed already in part 2. It was established that temperature differences between the three sites were insignificant, but on account of the differences in total rainfall, rainfall pattern and soil textures, soil moisture was not the same in the three locations It was concluded from the available data that during the period of grain filling (June in 1992) moistures were highest at Kildalton and lowest at Gusserane. The same barley samples were used for the determination of environmental effects on ß-glucanase as for the determination of the same effect on b-glucan, except they were micro-malted. Levels of ß-glucanase along with relevant statistical information at the three locations at different rates of applied nitrogen, for the varieties Blenheim, Pastoral and Puffin are shown in Table 5 Coefficients of variations of about two thirds of the listed values are considerably higher than those obtained for repeated determinations of the same sample It was shown in section 3 2 1 that poorer repeatability of separately micro-malted samples is due to the variability of micro-malting

According to the obtained results (Table 5) the lowest ß-glucanase levels were obtained at Oak Park for all three varieties at the three rates of applied nitrogen The highest ß-glucanase levels for Blenheim and Pastoral were obtained at Gusserane and Kildalton respectively at the three rates of applied nitrogen However, the highest ß-glucanase for variety Puffin was found at the low and medium rates at Gusserane, and at the high rate at Kildalton

It is not possible to establish from the data (Table 5a) the relative order of b-glucanase levels between Kildalton and Gusserane In four out of nine trials ß-glucanase levels were higher at Kildalton - and in the same number of trials higher at Gusserane Therefore, the only conclusion we can draw is that there is no difference

between the ß-glucanase levels obtained at the two sites

The only two locations between which significant differences in ß-glucanase levels were obtained for all three varieties were Oak Park and Kildalton at low rates of applied nitrogen (Table 5a). Differences between Oak Park and Kildalton at medium and high levels of nitrogen were significant in three out of six trials and between Oak Park and Gusserane at the three levels of nitrogen in three out of nine trials However in each case with one exception, in three out of nine trials, ß-glucanase levels are lower at Oak Park and the differences which are not significant are still relatively large. Most of these differences would probably be significant with improved precision Consequently it is possible to conclude that the lowest ß-glucanase levels were obtained at Oak Park.

Among the different environmental factors only the July rainfall is markedly different at Oak Park, it is 27 and 44% lower than at Gusserane and Kildalton respectively (Tables 17, Chapter 2) Grain maturing takes place during July, and given the same temperatures, the rate of gram maturing is by and large inversely proportional to the amount of rainfall Therefore it is possible that the rate of gram maturing will influence the grain's subsequent ability to produce ß-glucanase, and faster maturing such as at Oak Park will lead to decreased ß-glucanase production However, as the bio-synthesis of ß-glucanase is not understood at present it is not possible to explain what effect does the rate of grain maturing have on the dormant biochemical pathways which will be activated to produce ß-glucanase during germination.

3.3.3 Prediction of malt ß-glucanase levels from barley analysis

The possibility of predicting ß-glucanase levels in malted barley from either ß-glucan or ß-glucanase levels in wholegrain barley was evaluated If determination of malted barley ß-glucanase levels from unmalted barley were possible - there would be no need to go through the very time consuming process of micro-malting each sample

Linear regressions were carried out in order to establish if there is any correlation between ß-glucanase levels in malted barley and the ß-glucan levels of the corresponding barley ß-glucan levels of the varieties Blenheim, Pastoral and Puffin at three different locations and three rates of applied nitrogen and of all the varieties in the Teagasc and Departement of Agriculture trials, were correlated with the corresponding ß-glucanase levels Both of these sets of values show a wide range of ß-glucan and ß-

Table 5: Differences between ß-glucanase levels - environmental influences

Variety	Blenheim			Pastoral			Puffin		
Location	OP	KD	GUS	OP	KD	GUS	OP	KD	GUS
Low Nitrogen									
ß-glucanase levels (U/kg) n = 6	397.4	492.9	540.5	237.9	376.7	319.7	330.9	422.3	442.3
s.d.	53.7	27.7	28.1	37.6	81.5	81.5	39.0	78.1	104.5
CV (%)	13.5	5.6	5.2	15.8	21.6	30.8	11.7	18.5	22.3
Normal nitrogen									
ß-glucanase levels (U/kg) n = 6	365.5	444.5	482.2	308.3	331.2	231.8	330.5	415.4	458.5
s.d.	113.7	86.3	80.7	96.9	62.2	88.8	95.7	55.5	103.7
CV (%)	31.1	19.4	16.7	31.4	18.8	38.3	7.8	13.4	22.6
High nitrogen									
ß-glucanase levels (U/kg) n = 6	442.2	448.1	448.1	250.9	380.9	269.2	332.9	444.1	394.0
s.d.	61.4	101.0	108.1	18.3	45.1	93.7	15.5	56.5	91.6
CV (%)	13.9	22.5	22.2	7.3	11.9	34.6	4.7	12.7	23.2

OP = Oak Park; KD = Kildalton; GUS = Gusserane

Table 5a: Differences between ß-glucanase levels - environmental influences

Difference in ß-glucanase level	Low applied nitrogen			Medium applied nitrogen			High applied nitrogen		
	Blenheim	Pastoral	Puffin	Blenheim	Pastoral	Puffin	Blenheim	Pastoral	Puffin
OP - KD	-95 6*	-178 8*	-91 4*	-79 0 n s	-22 9 n s	-84 8*	-5 9 n s	-130 0**	-111 3**
OP - GUS	-143 1**	-81 8 n s	-91 5**	-116 8 n s	76 5 n s	-128 03*	-43 8 n s	-18 18 n s	-61 0 n s
KD - GUS	-47 5*	57 0 n s	0 0 n s	-37 8 n s	99 4 n s	-43 11 n s	-37 9 n s.	111 8*	50 1 n.s.

OP = Oak Park, KD = Kildalton, GUS = Gusserane

* = difference significant at the 5% level

n s = no significant difference

glucanase levels. Regression parameters listed in Table 6 show that with one exception less than 50% of the variation is accounted for by the regression line (R^2 x 100). Moreover the regression equation is different for each regression, hence there is no single equation for the correlation between ß-glucan and ß-glucanase levels. Consequently it is not possible to predict ß-glucanase levels in malted barley from ß-glucan levels in the corresponding unmalted barley.

Found ß-glucanase levels in several barley varieties are shown in Table 7. They are considerably lower than in malted barleys because most ß-glucanase in barley is released during germination. The obtained correlation between barley and and the corresponding malt ß-glucanase levels is poor. The square of the correlation coefficient (R^2)indicated that only 23% of the variation is accounted for by the regression equation. Consequently ß-glucanase levels in barley cannot be used for the prediction of ß-glucanase levels in the corresponding malt.

3.3.4 Attempted determination of ß-glucanase by liquid chromatography

Attempts were also made to determine ß-glucanase levels in malt by HPLC using a chromatofocusing column. The peptide components of a malt extract, with ß-glucanase level of 626 U/kg (Blenheim) could be separated into two not fully resolved peaks, A and B (Figure 1) in the pH range of 5.7 - 4.0. Addition of bethaine to the equilibrium and eluent buffers did not improve the resolution.

Peaks A and B were collected and both showed ß-glucanase activity. However peak A also had the same retention time as barley ç-amylase, which is extracted along with ß-glucanase. Thus peak A probably represents a mixture of ß-glucanase and ç-amylase. It was shown before that two peaks with ß-glucanase activity could be obtained when a similar malt extract was passed through a CM - Sepharose column at pH =4.5 (McCleary,1986).

In order to establish if the chromatographic data can be used for the determination of ß-glucanase chromatograms samples of known levels of the same were obtained. Peak heights and peak areas of each of the two peaks and combined were correlated by linear regression with ß-glucanase levels determined by the dye labelled ß-glucan method. Initial results based on seven barley varieties indicated that there is a good correlation (correlation coefficient 0.93) between the height of peak B and the determined ß-glucanase levels. However, analysis of a further set of sixteen samples

Table 6: Correlation between ß-glucan and ß-glucanase levels

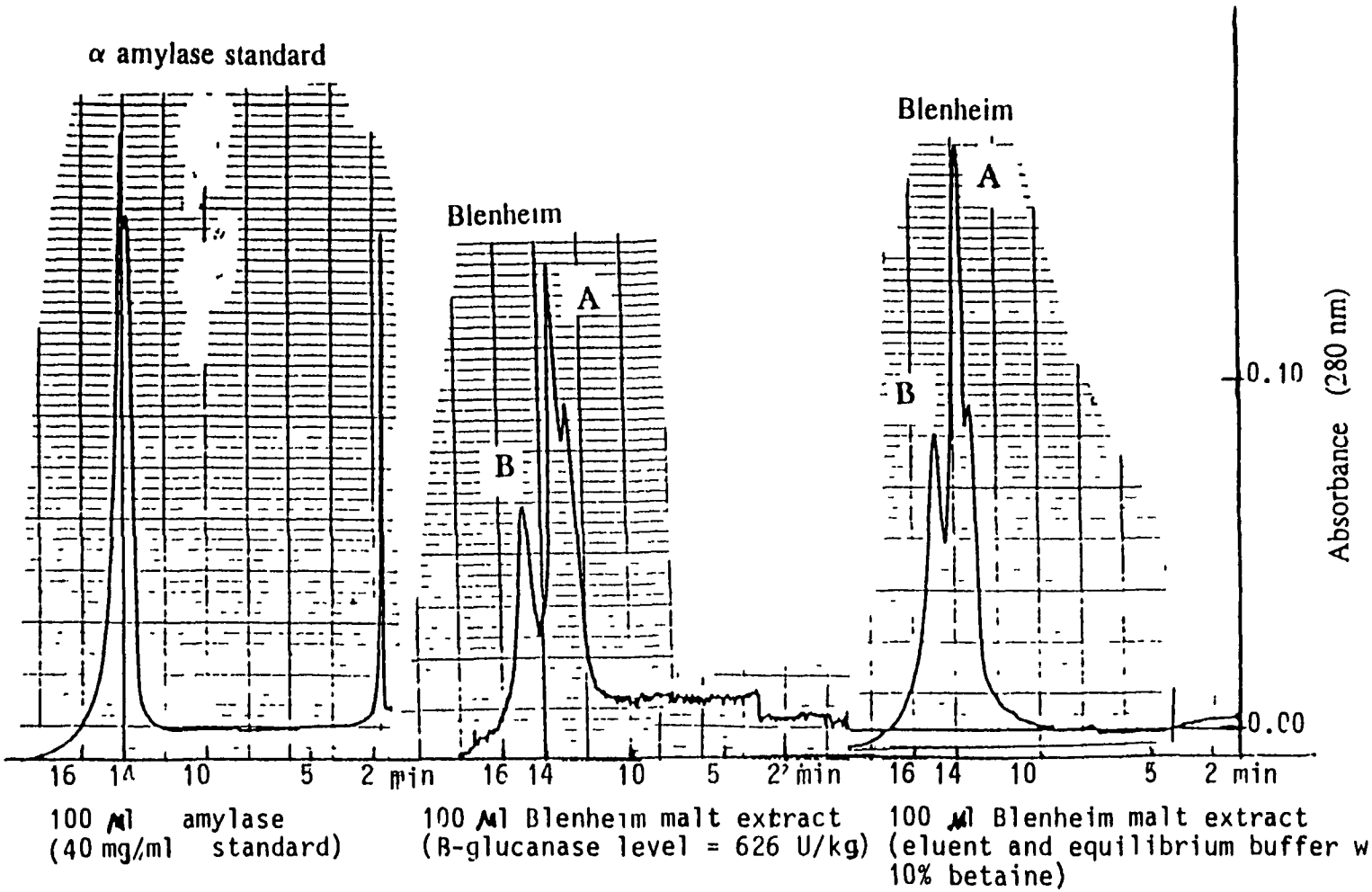
Barley	N	R	R^2%	% Regression equations	Tables
Blenheim	9	0 70	49%	y = -1 23 x + 7 39	3-3 vs. 3-2
Pastoral	9	0 75	56%	y = 0 44 x + 36	" " "
Puffin	9	0 09	1%	y = 0 04 x + 140	" " "
Total correlation	27	0 24	5%	y = 0.09 x + 134	" " "
Multivarietal correlation	12	0 30	9%	y = 0 18 x + 183	1-3 vs 3-2

u = number of values correlated,
y = ß-glucanase level,
x = ß-glucan level;
R = correlation co-efficient
R-sq% = fraction of variation explained by the regression line

Table 7: ß-glucanase levels in unmalted barley

Variety	Blenheim	Chad	Chef	Digger	Aisling	Grit
ß-glucanase level (u/kg) n = 6	18 20	17 97	17 31	16 57	17 18	19.1
s.d.	0.52	0 29	0.15	0.12	0 31	0.23
CV (%)	2.8	1 6	0 87	0.72	1 8	1 2

Figure 1 Separation of malt proteins by chromatofocusing



column, Mono P 5 x 200 mm; equilibrium buffer, 0.025 M bisTris, pH 7.1, eluting buffer Polybuffer 74, pH 4; flow 0.5 ml/min

of malt from four barley varieties grown in three locations with different rates of applied nitrogen, with ß-glucanase levels ranging from 191 to 580 kg/U showed a very poor correlation (correlation coefficient 0.34) Thus it was not possible to use the malt extract chromatogram obtained by chromatofocusmg for the determination of ß-glucanases.

A possible reason for the poor correlation between determined ß-glucanase levels and peak B is that each peak represents the response to several enzymes, rather than only ß-glucanases. We found that peak A also contained ç-amylase and McCleary, (1986), indicated that one of the peaks he isolated showed ß-glucosidase activity Changes in ß-glucanase levels do not necessarily bring about proportional changes in the levels of the other malt enzymes, hence poor correlation, peak parameters and one particular enzyme can be expected

3.5 CONCLUSIONS

The dye labelled - ß-glucan ß-glucanase assay was found to be convenient for the determination of ß-glucanase levels in a large number of malt samples and with careful techniques it is possible to achieve precisions below 10% However, the variability of micro-malting can increase the error of the ß-glucanase value by an additional 10-20%, which must be taken into consideration when levels of the same are compared Attempts to determine ß-glucanase levels in malt by chromatofocusing-HPLC or from barley ß-glucan or ß-glucanase levels were unsuccessful

There is a definite varietal effect on ß-glucanase as indicated by the fact that same order of ascending values Blenheim >Chad >Grit >Aisling, was constant in two consecutive years, and in two different environments. Environmental effects on ß-glucanase levels in malt independent of varietal effects have also been observed ß-glucanase levels for three varieties, Blenheim, Puffin and Pastoral were considerably lower at Oak Park than at the other two locations, although the specific reasons for the observed effects are far from clear Agronomic factors - such as sowing dates, had no observable effect on ß-glucanase levels

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