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HPLC AND IMMUNOCHEMICAL DETECTION OF GLIADIN IMPURITIES IN WHEAT AND WHEAT PRODUCTS

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in collaboration with

The Laboratory of the Government Chemist

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DECLARATION

This work has not been accepted in substance for any other degree, and is not concurrently being submitted in candidature for any other degree.

This is to certify that the work here was carried out by the candidate himself. Due acknowledgement has been made of all assistance received

Signed .....

(Candidate)

Signed .....

(Director of Studies)

TO JO

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ABSTRACT

HPLC AND IMMUNOCHEMICAL DETECTION OF GLIADIN IMPURITIES IN WHEAT AND WHEAT PRODUCTS

P.K. Mc CARTHY, 1990

Gliadin RP-HPLC of hexaploid wheat is shown to give "fingerprint type" profiles which are suitable for wheat variety identification. These profiles are found to reflect the genetic exchanges occuring during the crossing of wheat varieties in F1 hybrid wheat production. Two RP-HPLC techniques are proposed for F1 hybrid purity determination: the first distinguishes genetically different seeds types from F1 hybrid seed by their gliadin profiles and purity is presented as the percentage of F1 hybrid seeds in a mixture; the second utilises the gliadin RP-HPLC profile of a batch of seeds by comparing the peak area ratio of unique gliadin peaks to that obtained using standard blends corresponding to 100-50% hybrid purity. The total analysis time (2nd method) is less than four hours and the results compare favourably with those obtained using acid PAGE of gliadins.

When hexaploid wheat gliadins are separated by RP-HPLC under identical conditions a major doublet peak elutes at 47.20+/-0.33 min and 47.94+/-0.05 min which is absent in the gliadin RP-HPLC profiles of pure Durum wheat. This is used as the basis of a method to detect the level of common wheat adulteration in Durum wheat and pasta products. It has been found that protein denaturation occuring during high temperature processing leads to some distortion of the early eluting peaks of the RP-HPLC profile, but the area of interest for detection of common wheat adulteration of pasta is unaffected. For Durum samples containing low levels of adulteration electrophoresis of fractions collected from RP-HPLC is proposed as a means of improving sensitivity. It is shown that with the possible exception of acid PAGE of gliadins, the current methods used for detecting common wheat adulteration of pasta products, are inadequate. This effect is clearly seen in the alkaline PAGE and IEF patterns of polyphenoloxidases and esterases respectively, where the loss of enzymatic activity and resolution make it difficult to detect the presence or absence of common wheat in pastas dried at high temperature.

The biochemical characteristics (electrophoretic mobilities, 2-D PAGE patterns, isoelectric points and molecular weights) of the γ/β gliadins eluting between 47-49 min on RP-HPLC separation of hexaploid wheat specific gliadin are presented. Polyclonal antisera raised to γ-gliadin 44 and 46 (eluting between 47-49 min) cross-reacts specifically with γ-gliadins found in hexaploid wheat and Durum pasta adulterated with common wheat. An immunochemical method is proposed using this antibody preparation to identify traces of common wheat adulteration in gliadin extracts of Durum wheat or pasta electrophoretically separated and immobilised on nitrocellulose paper.

PUBLICATIONS

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CONTENTS PAGE

1.	INTRODUCTION	1
1.1.	WHEAT AND WHEAT PRODUCTS	1
1.1.1.	World Production	1
1.1.2.	Evolution of Wheat	2
1.1.3.	Cultivation of Wheat	5
1.1.4.	Hybrid Wheat	6
1.1.4.1.	Production of Hybrid Wheat Using Chemical Hybridising Agents	7
1.1.4.2.	Tests to Assess Hybrid Purity	8
1.1.4.3.	Certification of CHA Hybrid Seed	9
1.1.5.	Wheat Products	10
1.1.5.1.	Pasta Production	11
1.1.6.	Common Wheat Adulteration of Durum Wheat and Pasta	12
1.1.6.1.	U.K. Food Legislation	13
1.1.6.2.	E.C. Pasta Legislation	14
1.1.6.3.	Adulteration Detection	15
1.2.	COMPOSITION OF WHEAT FLOUR	18
1.2.1.	Wheat Protein	18
1.2.1.1.	Classification of Wheat Protein	19
1.2.1.2.	Cytoplasmic Protein	21
1.2.1.3.	Endosperm Storage Protein	24
1.2.1.4.	Genetic Constitution of Wheat Storage Protein	30
1.2.1.4.1.	Chromosomal Location of the Storage Protein Genes	32
1.3.	HIGH PERFORMANCE LIQUID CHROMATOGRAPHY	35
1.3.1.	Wheat Protein Preparation and Sample Application	35
1.3.2.	Temperature Effects on High-Performance Liquid-Chromatography	36
1.3.3.	High-Performance Liquid Chromatography Columns	37
1.3.4.	High-Performance Liquid Chromatography Separations	38
1.3.4.1.	Ion-Exchange High-Performance Liquid-Chromatography	38
1.3.4.2.	Size-Exclusion High-Performance Liquid-Chromatography	39
1.3.4.3.	Reversed-Phase High-Performance Liquid-Chromatography	39
1.3.5.	Advantages of High-Performance Liquid-Chromatography	41
1.3.6.	Limitations of High-Performance Liquid-Chromatography	43
1.4.	IMMUNOCHEMICAL ASPECTS OF CEREAL PROTEINS	45
1.4.1.	Polyclonal Antisera	46

1.4.2.	Monoclonal Antisera	48
1.4.3.	Conclusions	49
1.5.	AIM OF THIS STUDY	51
2.	MATERIALS AND METHODS	53
2.1.	MATERIALS	53
2.1.1.	Source of Chemicals	53
2.1.1.1.	Miscellaneous Items	55
2.1.2.	Laboratory Equipment	55
2.1.3.	Source of Wheat and Pasta	57
2.1.3.1.	F1 Hybrid Wheat	58
2.1.3.2.	Commercially Manufactured Pasta	58
2.1.3.3.	Manufacture of Laboratory Pasta	58
2.2.	ANIMALS	59
2.3.	EXTRACTION PROCEDURES FOR WHEAT PROTEINS AND STEROLS	60
2.3.1.	Gliadin Extraction	60
2.3.2.	Extraction of Polyphenoloxidases	61
2.3.3.	Extraction of Esterases	61
2.3.4.	Extraction of Wheat Sterols	62
2.4.	POLYACRYLAMIDE GEL ELECTROPHORESIS	63
2.4.1.	Acid Gel Electrophoresis of Wheat Gliadins	63
2.4.1.1.	Sample Preparation	63
2.4.1.2.	Solutions	63
2.4.1.3.	Gel Preparation and Electrophoresis	64
2.4.1.4.	Fixing Staining and Destaining of Gels	65
2.4.1.5.	Determination of Rm Values of Gliadins Separated by Acid PAGE	65
2.4.2.	Gel Electrophoresis of Wheat Polyphenoloxidases	66
2.4.2.1.	Sample Preparation	66
2.4.2.2.	Solutions	66
2.4.2.3.	Gel Preparation and Electrophoresis	67
2.4.2.4.	Staining for Polyphenoloxidase Activity	67
2.4.3.	Gradient SDS Polyacrylamide Gel Electrophoresis of Wheat Gliadins	68
2.4.3.1.	Solutions	68
2.4.3.2.	Sample preparation	69
2.4.3.3.	Gel Preparation and Electrophoresis	69
2.4.3.4.	Fixing Staining and Destaining of Gels	71
2.4.4.	Two Dimensional Electrophoresis	71
2.4.4.1.	Sample Preparation	72
2.4.4.2.	First Dimension Electrophoresis	72
2.4.4.3.	Second Dimension Electrophoresis	72
2.4.4.4.	Gel Staining and Destaining	73
2.4.5.	Gel Electrofocussing	73
2.4.5.1.	Electrofocussing of Wheat Esterases	73
2.4.5.1.1.	Sample Preparation	73
2.4.5.1.2.	Gel Preparation and Electrophoresis	73
2.4.5.1.3.	Staining for Esterase Activity	74

2.4.5.2.	Electrofocussing of Wheat Esterases	74
2.4.5.2.1.	Solutions	75
2.4.5.2.2.	Sample Preparation	75
2.4.5.2.3.	Gel Preparation and Electrophoresis	75
2.4.5.2.4.	Staining	76
2.5.	THIN LAYER CHROMATOGRAPHY	78
2.5.1.	Sample Preparation	78
2.5.2.	Preparation of TLC Plates	78
2.5.3.	Development of TLC Plates	78
2.6.	REVERSED PHASE HPLC	79
2.6.1.	Sample Preparation	79
2.6.2.	Elution Conditions	80
2.6.3.	Computation	80
2.7.	PROTEIN ESTIMATION ASSAY	81
2.8.	PRODUCTION OF POLYCLONAL ANTIBODIES	82
2.8.1.	Preparation of Antigen	82
2.8.2.	Immunisation Protocol	82
2.8.3.	Test Bleeding Monitoring and Immune Response	82
2.8.4.	Collection of Hyper-Immune Anti-Serum	83
2.9.	ENZYME LINKED IMMUNOSORBENT ASSAY	84
2.9.1.	Heterologous Non-Competitive Solid Phase ELISA	84
2.9.2.	Solutions	84
2.9.3.	Coating Wells with Gliadins	85
2.9.4.	ELISA Procedure	85
2.10.	ISOLATION OF ANTI-GLIADIN IgG	87
2.10.1.	Separation of IgG from Rabbit Anti-serum	87
2.10.2.	Preparation of Immunospecific Anti-γ-Gliadin IgG	87
2.10.2.1.	Coupling of D. Valdur Gliadins to Cn-Br Activated Sepharose 4B	87
2.10.2.2.	Immunoadsorbant Chromatography	88
2.10.2.3.	Chemical Elution of Bound Antibody	89
2.11.	ELECTROBLOTTING	90
2.12.	IMMUNOBLOTTING OF DURUM AND COMMON WHEAT GLIADINS	92
2.13.	STATISTICAL ANALYSIS	94
3.	RESULTS	95
3.1.	ANALYSIS OF GLIADINS BY RP-HPLC	97
3.1.1.	WHEAT CULTIVAR IDENTIFICATION	97

3.1.1.1.	RP-HPLC Separation of Gliadins	99
3.1.1.2.	Discussion	102
3.1.2.	ESTIMATION OF PURITY IN HYBRID WHEAT	106
3.1.2.1.	Production of F1 Hybrids	108
3.1.2.2.	Estimation of F1 Hybrid Purity Using Gliadin RP-HPLC Profiles	109
3.1.2.3.	Development of a Rapid RP-HPLC Method to Estimate Hybrid Purity	111
3.1.2.4.	Estimation of Hybrid Purity by RP-HPLC of Gliadins Extracted from Multi-seed Samples	116
3.1.2.5.	Discussion	119
3.1.3.	DETECTION AND QUANTIFICATION OF COMMON WHEAT ADULTERATION OF DURUM WHEAT	123
3.1.3.1.	Comparison of Gliadin RP-HPLC Profiles of Common and Durum Wheat	125
3.1.3.2.	Electrophoresis of RP-HPLC Separated Gliadins	127
3.1.3.3.	Detection of Common Wheat Adulteration of Durum Wheat using RP-HPLC	131
3.1.3.4.	Discussion	138
3.2.	EVALUATION OF RP-HPLC TO DETECT Triticum aestivum IN PASTA PRODUCTS DRIED AT HIGH TEMPERATURE; A COMPARISON WITH EXISTING METHODOLOGY	142
3.2.1.	Effects of Drying Temperature on Detection Methods	144
3.2.2.	Detection of Common Wheat Adulteration in Commercial Pasta Samples	151
3.2.2.1.	Adulteration Detection Using Electrophoresis Following RP-HPLC	159
3.2.2.2.	Reversed-Phase HPLC of Gliadins from Adulterated Pasta Samples Dried at High Temperatures	162
3.2.3.	Discussion	164
3.3.	CHARACTERISATION OF HEXAPLOID WHEAT SPECIFIC GLIADINS	168
3.3.1.	Determination of Electrophoretic Rm Values	170
3.3.2.	Characterisation of β-/γ-Gliadins	174
3.3.2.1.	Two Dimensional PAGE of Gliadins at Two pH Values	174
3.3.2.2.	Isoelectric Focussing of Gliadin Proteins	177
3.3.2.3.	Molecular Weight Determination of Gliadins	177
3.3.3.	Discussion	180
3.4.	DETECTION OF COMMON WHEAT ADULTERATION OF DURUM WHEAT AND PASTA BY IMMUNOCHEMICAL DETECTION OF γ-GLIADINS	184

3.4.1.	Modification of ELISA for Detection of Gliadins	186
3.4.1.1.	Immune Response to Gliadins	189
3.4.1.2.	Precipitation of IgG from Antisera	189
3.4.1.3.	Cross-reactivity of Anti-gliadin Antisera	191
3.4.2.	Isolation of Common Wheat Specific Anti-gliadin IgG by Indirect Affinity Purification	194
3.4.2.1.	Coupling of Durum Gliadins to Cn-Br Activated Sepharose 4B	194
3.4.2.2.	Assessment of Antibody Affinity and Purification of Anti-γ-Gliadin	194
3.4.2.3.	Assessment of the Cross-reactivity of Affinity Purified Anti-gliadin IgG.	195
3.4.2.4.	Interspecies Cross-reactivity of Anti-gliadin IgG	196
3.4.2.5.	Discussion	201
4.0.	GENERAL DISCUSSION	206
	REFERENCES	220

List of Tables

		Page
1.1	Polyphenol oxidase activities of wheat cultivars	23
1.2	Amino acid content (g/100g) of gliadin components	27
1.3	The Chromosomes of Wheat	31
1.4	Chromosomal Location of the Storage-Protein Genes of the Wheat Endosperm	33
3.1.	Mean+/-SE of Retention Times for Unique Gliadin Peaks used for Wheat Variety Identification	100
3.2.	Variation in the Occurence of Selected peaks used for Wheat Variety Identification	100
3.3	Relationship Between Peak Area Ratios (peaks e/b) and Percentage Hybrid Purity for Gliadin Extracts of a True F1 Hybrid and Standard Wheat Mixtures of Known Purity, Separated by RP-HPLC	117
3.4	Purity Analysis of F1 Hybrids SA 1-8 and SD 1-8	120
3.5.	Relationship between area of peaks eluting between 47 - 49 min and total protein applied to a RP-HPLC column	134
3.6	Detection of Common Wheat Adulteration of Pasta Samples Using Different Detection Procedures	152
3.7	Isolation of IgG from Rabbit Serum	192
4.1.	N-Terminal Sequences of Ponca Gliadins	215

List of Figures

		Page
1.1	The Evolution of Polyploid Wheats	4
1.2	Schematic Outline of Wheat Protein Extraction Protocol	20
1.3	A Diagramatic Representation of the Gliadin Banding Pattern of Bread and Durum Wheat Following Acid PAGE	26
3.1	RP-HPLC Separation Profiles of Gliadin Extracts from Hexaploid Wheat Varieties	100
3.2	Key to the Identification of 18 UK Hexaploid Wheat Varieties	103
3.3	Diagramatic Representation of the Field Trial Set-up used for Growing F1 Hybrid Wheat	108
3.4	Gliadin RP-HPLC Profiles of the F1 Hybrid Parents S♂ and S♀ and F1 Hybrids SA8 and SD8	110
3.5	Gliadin RP-HPLC Profiles of the Mixtures of F1 Hybrid Parents S♂ and S♀ Corresponding to Hybrid Purity between 100-50 Percent	114
3.6	Gliadin RP-HPLC Profiles of a True F1 Hybrid	115
3.7	Standard Curves Relating Peak Area Ratio and Percentage Hybrid Purity of Wheat Mixtures	118
3.8	RP-HPLC Separation Profiles of Gliadin Extracts from Hexaploid and Durum wheat varieties	126
3.9	RP-HPLC Separation Profiles of Gliadin Extracts from Durum Wheat Varieties	128
3.10	Electrophoretic Separation Patterns of Gliadins Collected During RP-HPLC Separation of Gliadin Extracts	130
3.11	RP-HPLC Separation Profiles of Durum Gliadin Extracts Containing (0-50%) Hexaploid Wheat	132
3.12	Electrophoretic Separation Pattern of Gliadin Extracts from Durum and Common Wheat Blends	136
3.13	Standard Curve Relating Peak Area Ratio with the Level of Hexaploid Wheat in a Durum Wheat Sample	137
3.14	Alkaline PAGE Separation Patterns of Specific Durum Wheat Polyphenoloxidases	145

3.15	Acid PAGE Separation Patterns of Alcohol Soluble Durum Wheat Gliadins	146
3.16	IEF Separation Patterns of Durum Wheat Water Soluble Esterases	148
3.17	TLC Separation Patterns of Durum Wheat Petroleum Ether Soluble Fatty Acid Sterols	149
3.18	RP-HPLC Separation Profiles of Durum Wheat Alcohol Soluble Gliadins	150
3.19	Alkaline PAGE Separation Patterns of Specific Polyphenoloxidases Extracted from Pasta Samples	153
3.20	IEF Separation Patterns of Water Soluble Esterases Extracted from Pasta Samples	154
3.21	Acid PAGE Separation Patterns of Alcohol Soluble Gliadins Extracted from Pasta Samples	156
3.22	TLC Separation Pattern of Pet-Ether Soluble Fatty Acid Sterols Extracted from Pasta Samples	157
3.23	RP-HPLC Separation Profiles of Alcohol Soluble Gliadins Extracted from Pasta Samples	158
3.24	Standard Curve Relating Peak Area Ratio with the Level of Hexaploid Wheat in a Pasta Sample	160
3.25	Electrophoretic Pattern of RP-HPLC Gliadins Extracted from Pasta 1 and Pasta 5	161
3.26	RP-HPLC Profiles of Gliadin Extracted from Pasta 1 and Pasta 5 before and after Drying at 85 ^{o}C	163
3.27	Determination of Gliadin Electrophoretic Mobility	171
3.28	Gliadins Electrophoretic Pattern of Unusual Hexaploid Wheat Varieties	173
3.29	Two-dimensional Electrophoretic Separation of Gliadins Extracted from Hexaploid Wheat	175
3.30	Isoelectric Focussing Patterns of Gliadins	178
3.31	Gliadin Molecular Weight Determination using Gradient SDS-PAGE	179
3.32	Titration curves of test sera obtained from rabbits immunised with total gliadins	188
3.33	Titration of Rabbit anti-gliadin serum by non-competitive ELISA	190
3.34	Immunoblots Produced after Incubation of Western Blots of Total Gliadin Extracts with Anti-gliadin Anti-sera	193

3.35 Immunoblots Produced after Incubation of Western Blots of Total Gliadin Extracts with Affinity Purified Anti-gliadin IgG 197

3.36 Immunoblots Produced after Incubation of Western Blots of Total Gliadin Extracts of European Hexaploid and Durum Wheat Varieties with Anti-γ-gliadin IgG 199

3.37 Immunoblots Produced after Incubation of Western Blots of Total Gliadin Extracts of 5 unusual European hexaploid wheat varieties with Anti-γ-gliadin IgG 200

3.38 Immunoblots Produced after Incubation of Western Blots of Total Gliadin Extracts of North American Durum Wheat varieties with Anti-γ-gliadin IgG 202

3.39 Immunoblots Produced after Incubation of Western Blots of Total Gliadin Extracts of 8 pastas Samples with Anti-γ-gliadin IgG 203

Abbreviations

CHA	- chemical hybridizing agent
CM	- chloroform-methanol
CMS	- cytoplasmic male sterility
DMAPN	- dimethylaminopropionitrile
DMSO	- dimethylsulphoxide
ELISA	- enzyme-linked immunosorbant assay
F1	- first generation
FDA	- Food and Drugs Administration
FPLC	- fast protein liquid chromatography
GSA	- general services administration
HIC	- hydrophobic interaction chromatography
HMM	- high molecular mass
HPLC	- high-performance liquid chromatography
IEF	- isoelectric focussing
LMM	- low molecular mass
LMMG	- low molecular mass gliadin
PAGE	- polyacrylamide gel electrophoresis
PBS	- phosphate buffered saline
RCF	- relative centrifugal force
RH	- relative humidity
SDS	- sodium dodecyl sulphate
TBS	- Tris buffered saline
TCA	- trichloroacetic acid
TEMED	- N,N,N',N',-tetramethylethylenediamine
TFA	- trifluoroacetic acid
tlc	- thin layer chromatography
Tris	- Tris(hydroxymethyl)-aminomethane
USDA	- United States department of agriculture

1. INTRODUCTION

1.1. WHEAT AND WHEAT PRODUCTS

1.1.1. World Production

Wheat provides about one fifth of all calories consumed by the human race. It accounts for nearly 30% of the world's grain production and for over 50% of the world grain trade. World wheat production more than doubled from 1950-1977, increasing from 172 to 382 million tonnes. During the same period world trade increased over 2.5 fold (from 28 to 73 million tonnes). World wheat production in 1986 increased to 450 million tonnes, of which about one fifth entered the world grain trade (Pomeranz, 1987a). The major hard red winter wheat producing areas are the U.S.A., Southern Eastern Russia, the Ukraine, Australia, China, Europe, Central India and Northern Africa. Australia and Argentina grow a medium hard wheat (9-13% protein). Hard red spring wheat is grown in Canada, the Northern States of the United States, the Soviet Union and China. The main soft wheat producing countries are, the European Community (E.C.), the U.S.A. and Australia (USDA, 1978). The major producing countries for tetraploid Durum wheat are Canada, the U.S.A., Argentina, the Soviet Union, North Africa and the EC (USDA, 1978).

Hexaploid wheats, the most commonly grown varieties (Menze et al., 1984) are divided into two groups: hard wheat in which the endosperm fractures neatly along the outlines of the cells into easily sifted particles; and soft wheat in which the endosperm fractures irregularily across the cells to produce a mass of fine debris with poor flow

properties. A state of confusion exists between European countries and the U.S.A. regarding the broad spectrum classification of wheat. In Europe all bread wheats (hard and soft) are called common wheat and the term "hard" is reserved for Durum wheats, whereas in the U.S.A. the term "hard" is used for bread wheats, "soft", for wheats for production of biscuits and cakes and Durum for wheats for production of alimentary pastas (NIAB, 1987). These wheats have a number of intervarietal and interspecies differences some of which can be explained by the evolutionary events which gave rise to modern day wheat.

1.1.2. Evolution of Wheat

Triticae Dumort (Hordeae Denth) is a tribe of the plant family poneaceae (Gramineae), containing three of the major cereals - barley, rye and wheat as well as the recently constructed cereal Triticale (wheat x rye) (Baum, 1977). The genus Aegilops L (wild grasses) is sometimes incorporated into this family, a classification which is not universally accepted (Morris and Sears, 1967). For the purposes of this report it will be treated as a separate genus. Despite the extensive volume of literature (reviewed by Baum 1977, 1978a, 1978b) available on the intergeneric and interspecific relationships in Triticeae spp., the taxonomy and nomenclature is still very confused (Dorofeev and Korovina, 1979).

The wheats form a polyploid series with diploid, tetraploid, and hexaploid species having 7, 14, 21 pairs of chromosomes respectively (Miller, 1987). Much of this evolution occurred in the wild.

(i Diploid Wheat

Diploid wheat is separated into four species, comprising a single genomic group with the genome formulae AA. This basic genome is common to all polyploid wheat (Johnson and Dhaliwal, 1976; Caldwell and Kasarda, 1978). Both wild (T Urartu and T boeoticum) and cultivated (T. Monococcum, T. Sinskajae) wheat are found in this group.

(ii Tetraploid Wheat

The tetraploid wheat are divided into two groups, with the genome formulaes AABB and AAGG. Again both wild and cultivated forms exist in each group, Figure 1.1). It is suspected by many workers (Pathak, 1940; Sears, 1956; Riley et al., 1958; Chapman et al., 1976; Feldman, 1978) that the B genome was transmitted during crossing of the diploid wheat within a species of the genus Aegilops (Figure 1.1). More recent studies show that chromosomes exist within Aegilops sitopsis which are preferentially transmitted and therefore always retained in the chromosome compliment (Maan, 1975; Miller et al., 1982). The transfer of these chromosomes could enhance the chance of obtaining a mixed genome (Miller, 1987). The donor of the G genome is suspected to be Ae speltoides (Shands and Kimber, 1973). This is supported by chromosome pairing studies (Shands and Kimber, 1973), cytoplasmic (Tsunewaki et al., 1980) and seed protein studies (Caldwell and Kasarda, 1978). However Gill and Kimber, (1974) showed using chromosome banding patterns that the G genome is very similar to but not identical to the S genome of Ae speltoides, thus casting doubt on the original

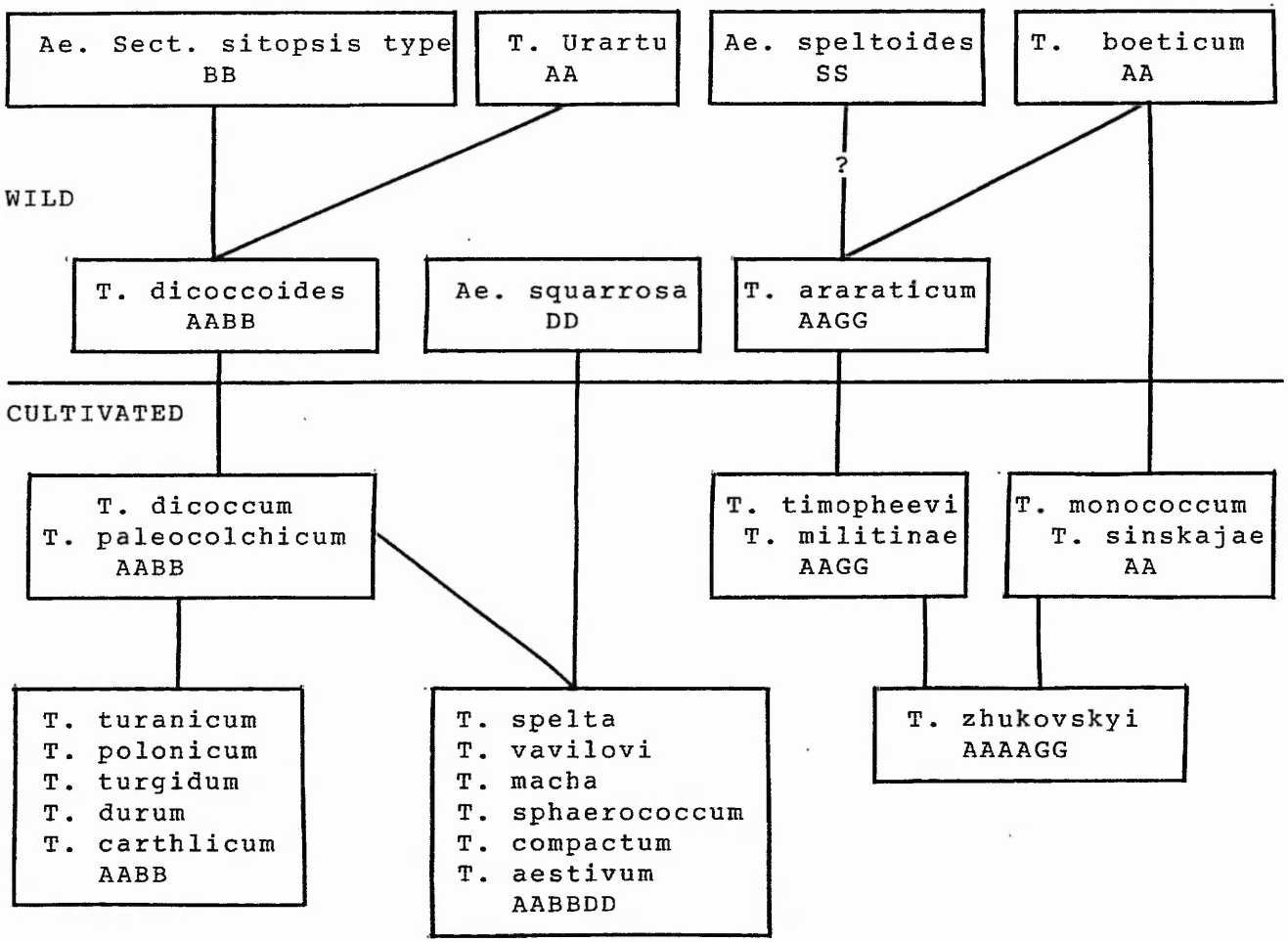


Figure 1.1 The evolution of polyploid wheats (from current evidence, Miller 1987).

hypothesis proposed by Shands and Kimber (1973).

iii) Hexaploid Wheat

The hexaploid wheats (Figure 1.1) are of two types AABBDD, and AAAAGG. By far the most important, is the AABBDD group, to which Triticum aestivum (common wheat) belong (Miller, 1987). It is now well established that these wheats are the result of amphiploidy between an AABB tetraploid wheat and the diploid D genome of Ae Squarrosa, (Pathak, 1940; Riley and Chapman, 1960). The hexaploids are all cultivated wheats (Figure 1.1,), no wild types exist.

1.1.3. Cultivation of Wheat

On the evidence of current archaeological finds, tetraploid wheats are believed to have been established by 8000 B.C. (Van Zeist, 1972; Van Zeist and Bakker-Heeres, 1979; Hillman, 1975; Mellaart, 1975) and hexaploid wheat by about 7000 B.C. (Hillman, 1972). Triticum dicoccum was probably the first variety cultivated, having grown in the wild it became established as a result of husbandry and artificial selection of naturally occuring wild wheat. The cultivated tetraploids grown today evolved from the initial form by a process of selection giving species such as the widely cultivated macaroni wheat T. durum (Muramatsu, 1986).

Not until the beginning of the 20th century was any major attempt made to improve the varieties of wheat crops grown. The crops grown at this time consisted of a wide range of land races, each of which had evolved in the area where it was grown mainly through natural selection. However as a result of widespread revived interest in Mendels laws, wheat breeding as we know it today began in

Britain and in many parts of the world (Percival, 1921).

Genetic improvements alone over the last 50 years have contributed to about half of the increase in wheat yields in Britain along with improving the disease resistance of the crop (Silvey, 1986). This has led to the government agencies providing "recommended variety lists" and schedules for controlling crop husbandry, fertilizer applications, and the use of herbicides, fungicides and pesticides.

In order to cope with the food requirements of the increasing world population along with meeting the challenge to reduce the need to import wheat of good breadmaking quality from countries like Canada further increases in the quality and quantity of wheat grown in Western Europe are required (Bingham and Lupton, 1987). Although wheat yield increases are still possible by controlled breeding programmes renewed interest has been shown in the production of F1 hybrid wheat varieties which can result in yield increases in the order of 15-25%.

1.1.4 Hybrid Wheat

Heterosis (hybrid vigour) in wheat was first reported by Freeman (1919) who showed that an F1 plant was generally taller than the taller parent. This encouraged the concept of producing hybrids in this naturally self-pollinating crop species. The first F1 wheat hybrids were grown after the successful development by Kihara (1951) and Fukasawa (1953) of cytoplasmic male sterility (CMS). Wilson and Ross (1962) developed a system of producing and maintaining male-sterile lines, as a result of which the first CMS engineered hybrid wheat was released in the U.S.A.

during 1974 and 1975. To date no CMS hybrid wheat has been found acceptable for European wheat production (Edwards, 1987).

Many problems are encountered during the production of CMS hybrid wheat, the most important being the problem of producing and maintaining male-sterile lines. This has prompted the development of chemical hybridising agents (CHA). Two such chemicals, from different sources have now been developed. In France the company Rohn and Moss developed a chemical in 1973 which inhibits pollen formation in the female designated plants. The oil company Shell, in 1979 discovered a chemical which operates by rendering the pollen incapable of fertilising the ovum. Using CHA technology, the female (male-sterile) parent can be any variety, thus removing the need to produce special male sterile stocks. To date CHA produced-hybrid wheat has been released in several countries including Australia, France, UK and U.S.A. (Edwards, 1987).

1.1.4.1. Production of Hybrid Wheat using Chemical Hybridising Agents

In a draft issued by the organisation of Economic Co-operation and Development (OECD), Hybrid Cereal Scheme, the following guidelines were proposed for the production of F1 hybrid wheat seed by use of CHA technology (Rutz, 1987): the F1 hybrid seed production field must consist of alternating strips of male and female parents, with a sufficient gap between the female and male strips and an area of pollination crop surrounding the field; the seed crop must be isolated by at least 25m from any other variety of the

same species except from a crop of the male pollen parent; the varietal purity of both parents is expected to be 99.7 percent; the hybrid seed producer must take all possible precautions to prevent admixture at harvest. Crop inspectors monitor the developing seed. In the first inspection they check that the above guidelines are implemented and in the second inspection they assess the level of male sterility in the female plants and the degree of varietal and species purity in the ripening grain.

1.1.4.2. Tests to Assess Hybrid Purity

In the OECD Hybrid Cereal Scheme two methods are approved for the assessment of varietal purity of F1 hybrid seed (Rutz 1987):

(a) Measurement of hybridity in the seed production field;

(b) A post-harvest electrophoretic test conducted before certification.

The hybridity can be assessed by placing pollen proof bags or pollen proof tents over a determined number of ears or plants of the female parent after CHA spraying and before anthesis. At the second field inspection the hybridity can be calculated according to the following formulae:

$$\text{Percentage of hybridity} = 100 \times \frac{(a-b)}{a}$$

where:

(a) is the number of fertilised grains in a sample of unprotected female seed parent and (b) is the number of fertilised grains in a specified number of ears sampled from the pollen proof bags or pollen proof tents.

Tents used in the seed production field can only

detect the extent of self-pollination taking place whereas electrophoresis, the post harvest control test can also detect pollination by foreign pollen. When electrophoresis is used it is necessary to examine individual seeds in a sample of 400 seeds in order to determine F1 hybrid purity (Feistritzer and Kelly, 1987). However Draper (1987) showed that acceptable results could be achieved using a 100 seed sample.

1.1.4.3. Certification of CHA Hybrid Seed

The inspectors responsible for assessing the purity of F1 hybrid seed must recognise that the methods used for checking non-hybrid varietal purity cannot be applied to F1 hybrid varieties (Rutz, 1987). The varietal homogeneity of these wheats is inferior to that of conventional varieties. This is due to the changing efficiency of the hybridising agent, resulting in a higher percentage of self-fertilised seeds. The purity, cannot be determined by routine field inspection, special pre- and post-harvest tests are required. In the directives of the E.C., on the marketing of cereal seed, only regulations for the certification of hybrid varieties of maize are described (Rutz, 1987). However it has been agreed between the E.C. and the international seed testing association (ISTA) that a minimum European F1 hybrid wheat varietal purity standard of 90 percent would be acceptable provided the hybrid variety satisfied the other criteria required for it to be registered as a new variety of wheat (Rutz, 1987). These criteria insist that the variety must be:

- morphologically or physiologically distinct

- stable to its description after propagation
- of value for cultivation and use and
- designated by a denominator suitable for registration.

At present the onus is on the certification authorities to verify the purity of F1 hybrid seed. In future as F1 hybrids become more popular this responsibility will pass to the seed producer, who will then have to ensure that official standards are maintained. The authorities have the task to control the marketed seed. Since producers are likely to have many different varieties of hybrid seed to certify automated and reliable analytical tests are required.

It is expected that the production of F1 hybrid wheat will increase over the next decade when it is realised that yield increases in the order of 15-25% more than compensates for the increased cost of using F1 hybrid seed. Furthermore the quality and functional properties of wheat flour for use in many products can be controlled, predicted and improved with the use of F1 hybrid wheat (Bingham and Lupton, 1987).

1.1.5. Wheat Products

Over two thirds of the annual world wheat crop is processed into flour for food. Common wheat flour is used to manufacture bread, biscuits and pastry products while Durum wheat flour is specifically used for the manufacture of pasta products. Industrial uses of wheat include the manufacture of malt, potable spirits, starch, gluten, pastes and core binders (Pomeranz, 1987b). In the U.S.A. small quantities of wheat flour (mainly low grade) are used to manufacture wheat

starch as a by-product of gluten. Gluten is used to supplement flour proteins in baked goods and as raw material for the manufacture of mono-sodium glutamate (Pomeranz, 1987b).

1.1.5.1. Pasta Production

i) Raw Material

The basic raw material for the production of high quality pasta is semolina milled from pure Durum wheat (Irvine, 1971). Sometimes egg powder is added and according to FDA standards of identity, egg noodles and egg spaghetti must contain a minimum of 5.5% egg solids. Optional ingredients include salt, enrichment (vitamins and minerals) and colour (US FDA, 1981). In some countries it is permissable to add specified quantities of common wheat, however in other countries this constitutes an adulteration.

ii) Manufacture

The first mechanical process to extrude pasta products dates back to the 19th century but continuous extrusion dates back only about 50 years and fully automated pasta production about 30 years (Iglett, 1974). The manufacture of pasta is a simple process: semolina from Durum wheat is mixed with water to form a dough (with approx 31% moisture), this on extrusion produces a translucent pasta product of acceptable cooking and eating quality (Grzybowski and Donnelly, 1979; Dexter et al., 1983); during the extrusion process the frictional heat generated in the extrusion barrels is dissipated by a water-cooled jacket fitted to the barrels thereby maintaining a constant extrusion temperature of approximately 55^{o}C. If the dough

becomes too hot (above 75°C) the quality of the finished product is severely decreased.

iii) Drying

Drying is without doubt the most difficult and critical step to control in the processing of pasta products. The objective is to lower the moisture content of the pasta from 31% to 12-13% humidity so that the product will be hard, retain its shape and will store without spoiling (Banasik, 1981; Hummel, 1966). Traditionally pasta was dried using a "normal" drying cycle. The moisture content of the extruded pasta is reduced from 31%RH to 25%RH in the pre-drier (zone 1). After completing zone 2 the moisture is reduced to 18%RH. Additional moisture is finally removed in zone 3. This process is thought to cause little or no structural or functional alteration in the molecular composition of the pasta. With modern processing equipment, many pastas are now dried in a "Superheat" drying cycle (0.5h at 90°C followed by 3.5h at 85°C). Two recent reports (Olivier, 1985; Manser, 1986) found no reduction in organoleptic quality for pastas dried at temperature greater than 100°C. Also it was shown that high temperatures drying improved the microbiological quality and reduced lysine losses in the pasta. However the effect of these denaturing conditions on the molecular composition of the pasta and hence on the methods used to detect common wheat adulteration in pasta remains to be clarified.

1.1.6. Common Wheat Adulteration of Durum Wheat and Pasta

Pasta is an important staple food for some member states of the EC. Italy consumes 25 kg/capita/year and

Greece, France and Spain approximately 6kg/capita/year (personal communication Dr. Autran). National legislation in these countries prohibits the manufacture of products made from common wheat or a mixture of common wheat and Durum wheat. In other countries (UK, Netherlands, Germany) where the per capita consumption is low and demand for quality is not so high, the use of common wheat flour is tolerated. However the best pasta (visual, organoleptic cooking quality and nutritional value) are processed from 100% Durum wheat semolina flour.

In a survey carried out in the Cheiti province of Italy between 1977 and 1986 (Sippio di and Trulli, 1987) it was shown that 50% of pasta tested was adulterated with common wheat. Of this 71% of samples contained between 2-4% common wheat and a further 21% between 5-10%. The remainder contained greater than 10% common wheat. However no obvious patterns of adulteration were reported.

1.1.6.1. UK Food Legislation

In the 1984 Food Act no special reference is made to the problem of common wheat adulteration of pasta. However part 1, sections 6 and 7 refer to the description of food in labelling and advertising. Section 6 may be paraphrased to state that it is an offence to label or advertise a food in a manner which,

(a) falsely describes the food

(b) is calculated to mislead as to its nature, or its substance or its quality.

Section 7 provides ministers with the power to regulate the manner in which food is described. It therefore becomes

clear that in the UK, pasta made from mixtures of common and Durum wheat can be traded, provided their composition is stated clearly on the label.

1.1.6.2. E.C. Pasta Legislation

Until recently countries who prohibited the manufacture and marketing of pasta made from common wheat and Durum/common wheat mixtures extended this prohibition to imported goods. The European court has now ruled (C335/15, 1985; C121/24, 1986; C215/09, 1988a; C215/10, 1988b) that this is incompatible with articles 30 and 36 of the treaty of Rome (1957). Article 30 states that:

> "Quantitative restrictions on imports and all measures having equivalent effect shall....be prohibited between member states."

This provision is subject to a number of exceptions which are set out in Article 36. The result of this ruling is to allow free circulation of common wheat pasta inside all member states. Price differentials between Durum wheat and common wheat will further increase this trend, creating an urgent necessity to differentiate between pasta prepared solely from Durum wheat and those prepared from Durum / common wheat blends. For export refund purposes the actual proportion of common wheat has to be established. Ultimately to satisfy consumer rights and enforce UK food legislation it is necessary to have an official method to determine the content of common wheat in pasta products. This method must be suitable for application to pastas manufactured in modern high temperature systems.

1.1.6.3 Adulteration Detection

Many interspecies differences between tetraploid and hexaploid wheats have been exploited by tlc, spectrophotometric, electrophoretic and immunochemical methods, in order to find a method suitable for detection of common wheat adulteration of pasta products.

i) Lipid Analysis

The earliest methods proposed for the detection of common wheat adulteration of pasta products (Matweef, 1952; Gillies and Young, 1964; Bernaert and Gruner, 1968), were based on the thin layer chromatographic (tlc) separation of phytosterol palmitate, a fatty acid sterol ester normally found in relative abundance in common wheat (Walde and Mangels, 1930) whilst only trace amounts are found in Durum wheat. Despite the criticisms expressed by several authors (Garcia-Faure et al., 1965; Custot et al., 1966; Fruchard et al., 1967) that this method lacks specificity for some common wheat which contain approximately equal levels of sitosterol palmitate as Durum wheat, it became the basis of the EC method (Anon, 1979), for the detection of common wheat in Durum pasta products for customs classification purposes. A further method based on the infrared spectroscopic detection of complex lipids, characteristic of common wheat (Brogioni and Franconi, 1963) has not proven to be successful in practice (Feillet and Kobrehel, 1974). The US recommended method uses a colour test based on the presence of the yellow carotenoid pigment in the pasta (GSA, 1975; AACC, 1983; USDA 1982). However the test is flawed because pasta adulterated with significent levels of common wheat has sufficient colour

to pass the test. Furthermore, wheat cultivars vary in pigment content which further complicates the test (Burgoon et al., 1985).

(ii Electrophoretic Analysis

A number of electrophoretic methods involving the identification of specific proteins characteristic of common wheat have also been used. The isoelectric focussing technique introduced by Resmini (1968) is based on the quantitative assessment of the ratio between focussed protein bands which are soluble in 0.8M $(NH_4)_2SO_4$ and precipitated by 1.2M $(NH_4)_2SO_4$. These protein bands are characteristic of common wheat and are absent in Durum wheat. It is reported that this procedure can detect adulteration of Durum wheat products down to 1% (m/m). A similar method used to separate wheat esterases was reported much later by Ainsworth et al. (1984) and Cooke et al. (1986), who used the method to differenciate Durum wheat and common wheat on their esterase content. Apart from this use no further application of this method is found in the literature.

According to the method of Feillet and Kobrehel (1974) the amount of common wheat in a Durum wheat or pasta product is estimated using polyacrylamide gel electrophoresis and densitometry of relative amounts of a polyphenoloxidase band of protein unique to common wheat. It is claimed that the genetic or agronomic origin of wheat, extraction rate, pasta drying temperatures of up to 70°C, or the presence of egg do not modify the results. Also it is claimed that the method can detect the presence of 3% (m/m) common wheat in a Durum wheat product.

The methods of Kobrehel et al. (1985) and Dysseller et al. (1986) are based on the gel electrophoretic identification of common wheat specific ω-gliadins. Wrigley and Shepherd (1973) have shown that ω-gliadins are encoded by the D genome, and hence are not found in tetraploid wheat. However no sensitivity limits were reported for these methods.

(iii Immunochemical Analysis

Immunochemical methods have also been proposed using antisera to wheat globulin (Piazzi and Cantagalli, 1969) and wheat albumin (Piazzi et al., 1972). Antibodies raised in rabbit and goat respectively have been used to assay for common wheat impurities in Durum wheat. Using immunodiffusion precipitation tests, wheat globulin antisera and wheat albumin antisera were reported to detect common wheat levels of 10% and 5% respectively. However the susceptibility of globulin and albumin cereal proteins to changes resulting from environmental growth conditions (Wrigley and Shepherd, 1973) casts doubt on the reliability of this method.

More recently a method was described by Pompucci et al. (1978) and Cantagalli et al. (1979) which used antisera raised to wheat germ lectin extracted from soft wheat (Allen et al., 1973). However later work by Bracciali et al. (1980) showed that a similar protein existed in Durum wheat and that antibodies raised against this protein cross-reacted with lectin from soft wheat grain.

1.2 COMPOSITION OF WHEAT FLOUR

The chemical composition of the dry matter of different cereal grains varies widely. Variations are found in the relative amounts of proteins (8-13%), lipids (1.4 - 2.0%), carbohydrates (70-75%), pigments, vitamins and ash (Simmonds, 1978). These components are not uniformly distributed in the different kernel structures which leads to further variations in the composition of milled wheat flours. The protein content of wheat is the index most commonly used to determine the quality of wheat flour used for the manufacture of various foods, furthermore as it is under genetic control (Wrigley and Shepherd, 1973) it is also used for wheat speciation and variety identification. The wheat proteins will be discussed in relation to their potential in the determination of hybrid purity and in the detection of common wheat in Durum pasta products.

1.2.1. Wheat Protein

Wheat proteins are a very heterogenous group of proteins and as already shown (section 1.1.2.) are the products of large multigene families. Their solubilities are atypical of most other plant and animal proteins. This is especially true of the prolamins (the name derived from proline and amine, Kasarda *et al.*, 1976). The prolamins (gliadins and glutenins) have an unusual amino acid composition, rich in proline, glutamine and hydrophobic amino acids and deficient in acidic and basic amino acid residues (Ewart, 1967; Huebner *et al.*, 1967; Platt and Kasarda, 1971). Some wheat proteins interact noncovalently with endosperm constituents such as lipids and carbohydrates and associate

noncovalently through hydrogen or hydrophobic bonds or covalently through disulphides with each other to form high molecular mass (HMM) complexes (Mc Craig and Mc Calla, 1941; Olcott and Mecham, 1947; Lasztity et al., 1979). In spite of these difficulties the wheat proteins are now well understood.

1.2.1.1. Classification of Wheat Protein

Research on wheat proteins began with the announcement by Beccari (1745) that he had successfully separated a wheat protein which he called gluten. For the next 160 years, different workers studied the composition of gluten and also the water soluble wheat proteins. Eventually this led to the publication by Osborne (1907) of a comprehensive monograph of wheat proteins. This classification system remains largely unchanged today as shown in Figure 1.2. Two major groups of proteins are recognised: the cytoplasmic proteins (albumins, globulins) characterised by their solubility in aqueous salt solutions; and the endosperm storage proteins, which includes aqueous alcohol soluble proteins (gliadins, HMM gliadins); proteins insoluble in aqueous salt or alcoholic solutions but soluble in dilute acid and alkali solutions (LMM glutenins); and proteins subunits only solubilised with the use of SDS and 2-mercaptoethanol (HMM glutenins).

In theory this classification system seems adequate however in practice an amount of overlap occurs which causes confusion regarding the classification of some proteins. This is especially true for the endosperm proteins some of which belong to different groups but have very

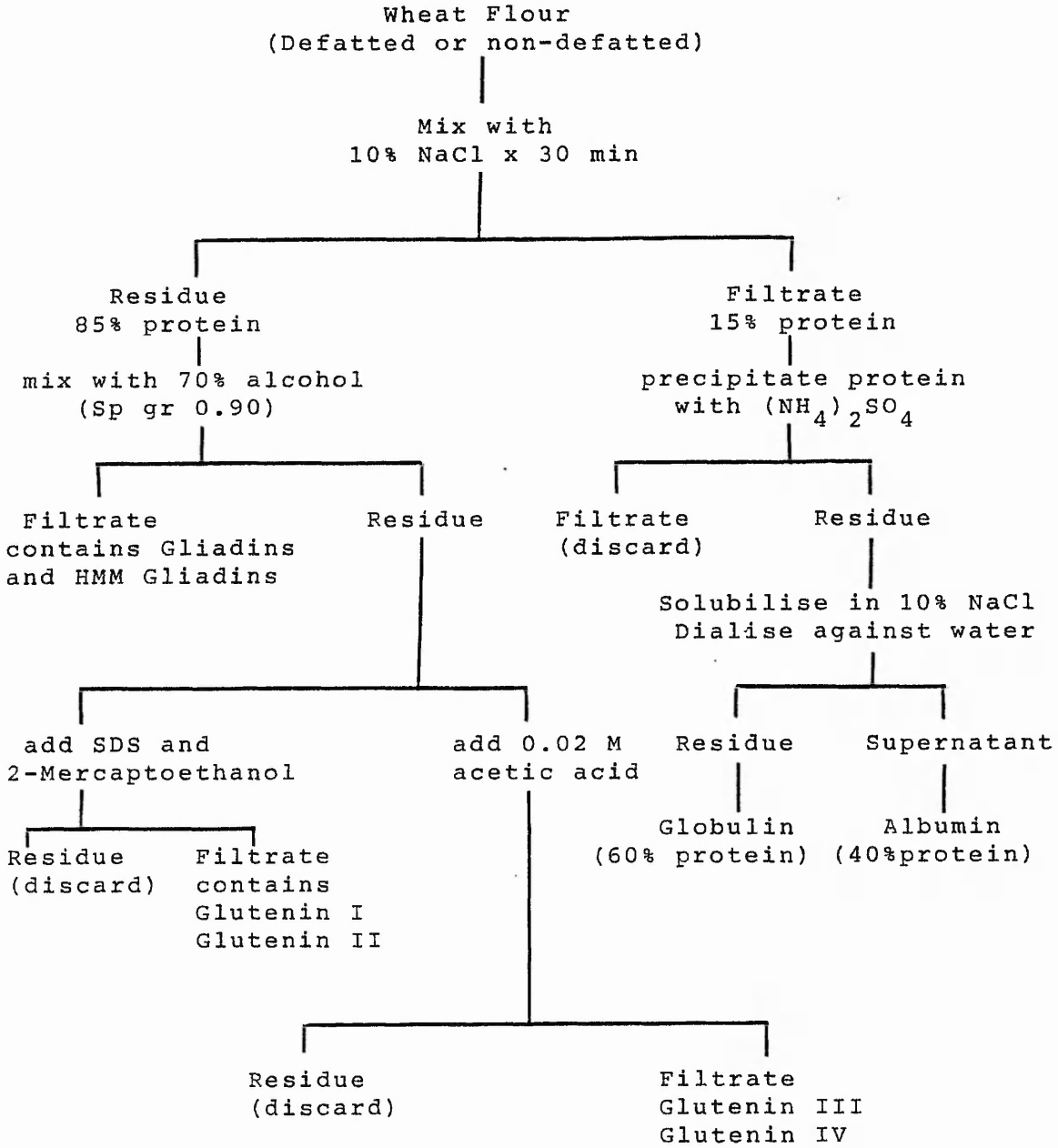


Figure 1.2 Schematic outline of wheat protein extraction protocol (Graveland et al., 1982)

similar biochemical characteristics (Charbonnier et al., 1980). Nevertheless consistent intervarietal and interspecies differences have been identified in wheat proteins (Lasztity, 1984). These differences and their uses along with the general properties of the proteins are discussed in the following sections.

1.2.1.2. Cytoplasmic Protein

Wheat albumins and globulins are readily extracted in salt solutions. While 0.5M NaCl will optimally extract about 30% of the total N, it also extracts about 5% of N comprising low molecular mass storage proteins (Miflin et al., 1983). The quantity extracted increases as the ionic strength of the solution increases. The lowest proportion of prolamin-type protein is extracted using phosphate and magnesium buffers (Lasztity, 1984).

It is generally accepted (Wrigley et al., 1982) that albumin and globulin fractions of wheat are poorly suited to wheat varietal identification. Nevertheless this varietal uniformity makes the proteins suitable for interspecies comparisons because of the compositional differences found in different species. Analysis by electrophoresis of this group of proteins has proved valuable in determining levels of Triticum aestivum in Durum wheat flour and in pasta products manufactured in low temperature systems (Resmini, 1968; Ainsworth et al., 1984; Cooke et al., 1986; Feillet and Kobrehel, 1974).

Kruger (1976) reported that, common wheats contain higher levels of polyphenoloxidase activity than the Durum wheats. The enzyme is concentrated in both the outer layers

and in the embryo of the grain. Using PAGE he detected as many as 12 isoenzymes. Intervarietal differences had previously been reported by Tikou et al. (1973) who showed that the diphenol catechol was a suitable substrate for measuring polyphenoloxidase activity. Lamkin et al. (1981) observed a significant difference between the levels found in 31 common and Durum wheat varieties (Table 1.1). This difference in polyphenoloxidase activity became the basis of a test developed by Feillet and Kobrehel (1974) to detect the presence of common wheat flour in Durum wheat semolina flour and pasta products dried below 70°C.

Meredith et al. (1960) extracted two cytoplasmic lipoproteins using either a methanol-chloroform mixture, or 2M urea. Then he showed that the amino acid composition of one of these proteins was similar to that of gliadin. However the other (lower molecular mass fraction) was composed of two clearly defined groups of hydrophobic proteins: CM proteins (Garcia-Olmedo and Garcia-Faure, 1969; Redman and Ewart, 1973; Salcedo et al., 1978a); and low molecular mass gliadins (LMMG) (Prada et al., 1982; Salcedo et al., 1979). Five components designated CM1, CM2, CM3, 16, 17 (Salcedo et al., 1978a) were found in hexaploid cultivars. In tetraploid wheat only an infrequent allelic variant of CM3 exists, (Salcedo et al., 1978a; Salcedo et al., 1978b). The molecular mass of the CM proteins is about 12,000 -13,000 (Garcia-Olmedo and Carbonero, 1970; Redman and Ewart, 1973; Salcedo et al., 1978a) and they contain a high proportion of hydrophobic amino acids (49-59% of total amino acid residues). They also contain lower amounts of glutamic acid

Table 1.1. Polyphenol oxidase activities of wheat cultivars Activity-oxygen per ml^{-1} min^{-1} (Lamkin et al., 1981)

Class and Cultivar	Catechol Activity
Hard red winter	
Eagle (1978)	28.9 +/- 1.3
Bucksin (1978)	28.2
Scout (1978)	25.3
Centurk (1978)	25.7
Parker (1977)	26.4
Soft red winter	
Abe (1978)	28.5
Arthur (1977)	21.4
Monon (1977)	18.1
Arthur 71 (1977)	26.7
Arthur 71 (1978)	25.0
Logan (1978)	15.3
Hard red spring	
Coteau (1978)	23.7
Olaf (1978)	17.5
Waldron (1977)	26.7
Era (1978)	15.4
Thatcher (1978)	20.4
Kitt (1978)	21.0
White common	
Luke (1977)	12.2
Nugainest (1977)	22.0
Ionia (1977)	21.1
Genesee (1977)	6.8
Hyslop (1975)	16.1
Club	
Paha (1977)	7.5
Moro (1977)	8.3
Omar (1975)	8.3
Barbee (1977)	15.7
Durum	
Roletta (1978)	4.5
Rugby (1978)	2.8
Ward (1978)	3.4
Calvin (1978)	3.8
Cando (1978)	4.1

and proline and a higher lysine content than gliadins.

More recently Greenwell and Schofield (1986) have identified low molecular mass (15K) starch granule proteins, extracted using SDS, these are present in significant quantities in "soft" wheat and to a lesser extent in "hard" wheat. The amino acid composition of these indicated similarities with water soluble proteins rather than endosperm proteins. Durum wheat completely lacks these proteins.

1.2.1.3. Endosperm Storage Protein

After extraction of the salt soluble proteins, about 85% of the total N remains, this comprises the seed storage proteins (Figure 1.2). Miflin et al. (1980) showed that 20% and 60% of this N could be extracted using 70% v/v aqueous ethanol and 50% v/v aqueous propan-1-ol, 1% v/v aqueous glacial acetic acid, 1% v/v 2-mercaptoethanol respectively. A polypeptide with a molecular mass of 100 kd was observed on the SDS PAGE pattern of the latter fraction and identified as HMM gliadin (Miflin et al., 1980). This is consistent with the earlier work of Dill and Alsburg (1925) who showed that a proportion of gliadins in alcoholic solutions precipitate at and around 4^{o}C. Danno et al. (1974) and Orth and Bushuk (1973) showed some of the remaining protein could be extracted with 0.01-0.05 M acetic acid and this was later called glutenin III and glutenin IV by Graveland et al. 1982. The remaining fraction includes the high molecular mass glutenin subunits (Glutenin I and Glutenin II) which were extracted by Burnouf and Bitz (1989) using a phosphate buffer containing SDS and 2-mercaptoethanol

after pre-extraction with 90-100% dimethylsulphoxide, of the non-glutenin part of the flour.

i) Gliadin Protein

The gliadin proteins of wheat can be categorized broadly into 2 groups, (Shewry et al., 1986) on the basis of being either sulphur rich (HMM gliadins, α,β,γ-gliadins, Figure 1.3) or sulphur deficient (ω-gliadins) or three discrete groups (α/β, γ, and ω) on the basis of N-terminal sequencing studies (Shewry and Miflin, 1985; Laztity, 1984; Kreis et al., 1985; Autran et al., 1979).

Amino Acid analysis (Charbonnier et al., 1980) shows (Table 1.2) that all gliadins have high contents of proline and glutamic acid, (>50% in ω-gliadins), which is present mainly as glutamine. Glutamine provides a concentrated source of N, that can be used by the germinating seed Lasztity (1984). Gliadins are deficient in lysine, arginine and histidine which reduces their human nutritional value. The low level of free carboxyl groups also makes it one of the least charged proteins known.

The molecular mass of the gliadin components (established using ultracentrifugation, SDS PAGE and gel filtration) indicates that the α β, γ-gliadins have molecular masses between 30 and 40 Kd (Sexson et al., 1978), ω-gliadins about 60 Kd and the HMM gliadins about 100 Kd (Hamauzu and Yonezawa, 1978). The molecular structure is considered to be a globular conformation with a low proportion of α-helix which is probably as a result of the high levels of proline (Lasztity, 1984). Disulphide bonds are intramolecular and hydrophobic interactions occur between the different

Figure 1.3. A Diagramatic Representation of the Gliadin Banding Patterns of Bread Wheat 1, Durum Wheat 2, following Acid PAGE

Table 1.2 Amino acid content (g/100g) of gliadin components (according to Charbonnier et al., 1980).

Amino Acid Groups	Gliadins					
	α	β	γ	ω_1	ω_2	ω_3
Basic Amino Acids	5.2-5.9	4.5-4.9	3.6-4.4	3.2	2.3-2.5	1.0-1.4
Glutamic Acid and Glutamine	33-39	34-40	38-40	49	56.0	45.0
Arginine	15-16	14-16	9-10	2.0	2.0-2.5	2.0- 2.5
Proline	15-16	15-17	16-19	20	19 - 20	25 - 30
Cysteine	2.0-2.6	2.2-2.6	2.0-2.5	1.0	0	0
Methionine	0.7-1.0	0.6-1.4	0.8-1.6	0.3	0	0
Phenylalanine	3.5-4.0	3.3-4.9	4.0-5.5	8.5	9.5-10.3	8.8-9.4

subunits, (Lasztity et al., 1976; Varga, 1978).

The presence of surface hydrophobicity in the gliadin subunits has been confirmed by the work of Popineau et al. (1980), Popineau and Godon (1982) and Popineau (1985) using hydrophobic interaction chromatography on phenyl and octyl sepharose CL 4B. It was shown that ω-gliadins have low and γ-gliadins have high levels of surface hydrophobicities, while α- and β gliadins are more heterogenous with some proteins having high and others low hydrophobicities. Similar results were obtained by Bietz and Burnouf (1985), Popineau and Pineau (1987) and Lookhart and Albers (1988) using RP-HPLC separation of total gliadin extracts.

No correlations have yet been found to link breadmaking quality in hexaploid wheat and the presence of particular gliadin proteins (Bramlard and Dardenet, 1985). A possible relationship has been shown between the ratio of certain gliadins and glutenins and good breadmaking quality (Huebner and Bietz, 1986). However Damidaux et al. (1978; 1980) using acid PAGE of Durum wheat gliadins found γ-gliadin bands, designated γ45 and γ42 according to the nomenclature of Bushuk and Zillman (1978), which appear to be correlated with gluten strength (viscoelasticity) and gluten weakness respectively. From 117 varieties of Durum wheat analysed, 66 displayed γ45 and 47 displayed γ42, 4 varieties could not be classified into either group. This relationship was confirmed by Kosmolak et al. (1980), du Cros et al. (1982) and more recently by Burnouf and Bietz (1984a) using RP-HPLC. Interestingly Cottenet et al. (1983; 1984) showed that γ42 and γ45 had identical molecular masses (45,000) and only

small differences in amino acid composition and surface hydrophobicity. These differences are insufficient to explain entirely the opposite effect exhibited by these proteins on gluten strength.

Gliadin toxicity (coeliac disease) is now well established and studies have shown the following order or toxicity. α gliadins > β gliadins > γ gliadins (Kendall et al., 1972; Evans and Patey, 1974; Patey et al., 1975; Jos et al., 1979). Only ω-gliadins have been shown to be unnoxious.

ii) High Molecular Mass Storage Protein

The high molecular mass storage proteins, comprising Glutenins I, II, III, and IV, (Figure 1.2) are characterised by their insolubility in salt solutions and aqueous alcohol solutions (Lasztity, 1984). However as already discussed glutenin I and II can be extracted using SDS and 2-mercaptoethanol and glutenin III and IV can be extracted using 0.01-0.05 M acetic acid (Burnouf and Bietz, 1989; Graveland et al., 1982).

SDS PAGE of solubilised glutenin (I -IV) reveals that it contains 17-20 subunits, with low (approx 10-70 Kd) and high (80-130 Kd), molecular masses, glutenins III and IV and glutenins I and II respectively. These subunits are joined by disulphide bonds to form insoluble high molecular mass complexes (Bietz and Wall, 1972; Orth and Bushuk, 1973 and Orsi and Meresz, 1982). All glutenin subunits from the hexaploid wheat have similar amino acid compositions which are also similar to the amino acid composition of gliadins (Graveland, 1980; Graveland et al., 1982; Ryadchikow et al., 1980). However significant differences are observed between

the amino acid composition of glutenin preparations (prepared by the same method), obtained from tetraploid and hexaploid wheat (Orth and Bushuk, 1973; Wasik and Bushuk, 1974).

1.2.1.4. Genetic Constitution of Wheat Storage Protein

The three genomes of bread wheat (Triticum aestivum) are known to be closely related (section 1.1.2), with the original genome donors being diploid species of Triticum and Aegilops (Riley, 1965). Each genome consists of seven pairs of chromosomes giving a total of 21 pairs in the hexaploid-wheat nuclei (Table 1.3). Each chromosome pair is designated a number followed by a letter, the letter refers to the genome from which the pair was inherited. Chromosomes from different genomes that are genetically similar (or homoeologous) to each other are given the same number, for instance chromosomes 1A, 1B and 1D. The A, B, and D genomes encode sets of storage proteins that are electrophoretically distinguishable from each other, because of mutation of their structural genes since the divergence of the common ancestor of the A, B, and D genome donors (Payne et al., 1984a). It follows therefore that bread wheat will have many more storage protein components than related diploid species such as barley (Hordeum Vulgare) or tetraploid wheat (Triticum Durum). As the chromosomes in hexaploid wheat are effectively triplicated, partial or whole chromosome deletions are usually not lethal, for essential genes on eliminated chromosomes will be present on their homoeologues.

Table 1.3 The Chromosomes of Wheat (from Payne *et al.*, 1984a).

Possible Donor Chromosome	A genome Triticum urartu	B genome Ae. sitopsis	D genome Ae.squarrosa
1	1A	1B	1D
2	2A	2B	2D
3	3A	3B	3D
4	4A	4B	4D
5	5A	5B	5D
6	6A	6B	6D
7	7A	7B	7D

1.2.1.4.1. Chromosomal Location of the Storage Protein Genes

The first major contribution to gene location was published by Shepherd (1968), who studied the gliadin proteins of nulli-tetra and ditelocentric lines of Chinese Spring by starch gel electrophoresis at pH 3.2 using an aluminium lactate buffer. On the basis of presence and absence of electrophoretic bands in different stock, genes for nine gliadins were shown to be located on chromosomes 1AS, 1BS, 1DS, 6AS, and 6DS. The remaining electrophoretic bands did not vary in any of the genetic lines, presumably because they consisted of mixtures of gliadins whose genes were located on different chromosomes.

Wrigley and Shepherd (1973) improved greatly the resolution of gliadins by the application of two dimensional electrophoresis, isoelectric focusing (I.E.F.) in the first dimension and starch gel electrophoresis at pH 3.2 in the second. They showed that all the gliadin protein genes were located on either chromosomes 1A, 1B, 1D, 6A, 6B, or 6D. These results were confirmed recently by Payne et al. (1982), who used a different two dimensional system, polyacrylamide gel electrophoresis at pH 3.1 followed by a sodium dodecyl sulphate PAGE separation in the second dimension. Both groups showed that all the ω-gliadins, most of the γ-gliadins and a few of the β-gliadins are controlled by the group 1 chromosomes whereas all the α-gliadins, most of the β-gliadins and a few γ-gliadins are coded on the group 6 chromosomes. In addition, Payne et al. (1982) showed that all the genes occurred on the short arms of the homoeologous group 1 and group 6 chromosomes (Table 1.4).

Table 1.4 Chromosomal Location of the Storage-Protein Genes of the Wheat Endosperm (from Payne *et al.*, 1984a).

Gene Locus*	Chromosome	Arm	Position	Storage Proteins
Glu-A1	1A	Long	close to centromere	HMM-Glutenin
Glu-B1	1B			
Glu-D1	1D			
Gli-A1	1A	Short	towards the end	ω-,γ-Gliadins
Gli-B1	1B			β-gliadins (few)
Gli-D1	1D			LMM-Glutenin
Gli-A2	6A	Short	towards the end	α-,β-Gliadins
Gli-B2	6B			γ-gliadins (few)
Gli-D2	6D			

* the three homologous sets of loci are called collectively Glu-1, Gli-1 and Gli-2

These findings suggest that the total possible number of protein combinations which can occur in a single variety is in the order of 3×10^7 which is far more than the number of varieties registered in the world collection of wheat (Blackman and Payne 1987). It has been proposed that this allelic variation in protein type accounts for varietal differences in the quality of wheat protein for bread and pasta manufacture. It further serves to highlight the major differences in the protein sub-composition of glutenin and gliadin extracted from hexaploid (AABBDD), and tetraploid (AABB) wheats.

1.3 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Since it was first introduced by Ackers (1970) high performance liquid chromatography (HPLC) has developed into a sophisticated chromatographic technique, capable of separating complex biological mixtures (Bietz, 1985a). Its success is mainly due to the major improvements made over the last twenty years in instrumentation and column design. These improvements have resulted in the development of solvent delivery systems capable of maintaining accurate and constant flow rates (0.1-10ml min^{-1}) and gradient formers which vary pump voltage with time, thus permitting the delivery of linear and non-linear gradients (Macrae, 1988). In many recent instruments, a system controller can form gradients, control an autosampler and detector and aquire and process data with the aid of a micro-computer. Advanced systems use personal computers to operate data aquisition, storing raw data for subsequent reintegration, replotting and comparison (Macrae, 1988).

1.3.1. Wheat Protein Preparation and Sample Application

A number of solvents suitable for HPLC analysis, including the chromatographic elution solvents have been used for cereal protein extraction and solubilisation (Bietz, 1979; Bietz *et al.*, 1984a; Bietz and Cobb, 1985; Kruger *et al.*, 1988). For column stability solvents should generally be between pH 2.0 and pH 8.0, the pH range in which silica particles are stable.

Bietz *et al.* (1984a) determined optimal conditions for extraction of wheat gliadin for HPLC varietal identification. Defatting and prior NaCl extraction were

considered unnecessary. A 30 min extraction in 70% v/v aqueous ethanol or 55% v/v aqueous 2-propanol were found most suitable. Extracts were found to be stable for at least one month at room temperature but protein association or precipitation occured if samples were frozen. Solvents such as lactate buffers, 2M urea and 2M dimethylformamide are less acceptable than aqueous alcohols, because they extract non-gliadin proteins, including native HMM glutenins which may aggregate on standing due to the formation of disulphide cross linking. This can ultimately lead to poor resolution in the resulting chromatogram (Bietz, 1983).

Extraction procedures for cereal proteins were also modified to cater for other HPLC techniques. Batey (1984) extracted wheat gliadins with 70% v/v aqueous ethanol (following preliminary salt extraction) and after freeze drying the extract, resuspended the protein in the initial ion exchange HPLC (IE-HPLC) solvent. For size exclusion HPLC (SE-HPLC) (Bietz, 1984a), samples were dissolved in column eluent buffer (0.1% sodium phosphate SDS pH 7.0, and 1% 2-mercaptoethanol).

1.3.2. Temperature Effects in High Performance Liquid Chromatography

Varying the column temperature between 20°C and 40°C was found to have little effect on RP-HPLC separation of wheat proteins (Bietz, 1983), however constant temperature must be maintained to maximise reproducibility. The chromatographic resolution improved dramatically when the temperature was raised from 20°C to 70°C (Bietz, 1984b; 1986; Bietz and Cobb, 1985). This is possibly due to either a

decrease in the extent of hydrogen bonding (Cole et al., 1983) or stronger hydrophobic interactions (Tanford, 1973) with the stationary phase at the higher temperature.

1.3.3. High Performance Liquid Chromatography Columns

Central to the separation is the chromatographic column, the design and ultimately the performance of which has improved dramatically over the past 20 years. Analytical columns are generally made of stainless steel (glass and plastic lined columns are also available), with an internal diameter of 4.0-4.6mm and 100-250mm in length. Initially these columns were packed with small silica particles (pore size of between 80-100Å) which were only suitable for separating low molecular mass proteins (<15000 Kda). More recently columns suitable for high molecular mass proteins have become available (Regnier and Gooding, 1980; Hearn et al., 1982). These columns, generally designated "large pore" or "wide pore" columns contain microparticulate silica packings (particle size 3-10 μm) with pore size of 300Å, exposing a greater surface area of silica for contact with polypeptides of molecular masses up to several thousands. The attachment of high loadings of bonded phase (carbon loadings) to the silica silanol groups plus the deactivation of most remaining free silanols by "end capping" (reaction of a small silylating agent) gives these columns a high capacity for proteins and an excellent recovery of bound protein. Furthermore various bonded phases may be covalently attached to the silanol groups, resulting in reversed phase (RP), ion exchange (IE) and size exclusion (SE) HPLC columns.

Columns are generally stable for hundreds of

separations provided they are maintained in solutions between pH 2-8. The use of inline filters and guard columns also aid in the protection of the column from particulates and solutes that bind irreversibly to the packing (Bietz, 1985a). Although most columns are expensive to buy, the cost per analysis is low when compared to that of other chromatographic techniques. The savings in analysis time can however justify their use.

1.3.4. High Performance Liquid Chromatography Separations

1.3.4.1. Ion-Exchange High Performance Liquid Chromatography

Numerous silica based cation-/ anion-exchange HPLC columns have been developed (Regnier and Gooding 1980; Hearn *et al.*, 1982; Bietz, 1985a) and applied extensively to noncereal proteins. Attempts to apply these to wheat endosperm storage proteins have met with limited success (Bietz 1985a). However Wingad *et al.* (1986) showed that barley hordein proteins could be separated by anion-exchange HPLC, revealing 13 component peaks. Both resolution and reproducibility were excellent indicating that the technique offers considerable potential as a method for barley cultivar identification. Batey (1984) using an IE-FPLC mono Q column (in which the stationary phase is a composite of hydrophillic polyether, monobeads, modified to have ion-exchange functionality) reported good separation of gliadins over 20 min and demonstrated that most wheat varieties could be distinguished using this technique.

1.3.4.2. Size-Exclusion High Performance Liquid Chromatography

Numerous silica-based SE-HPLC columns suitable for separating proteins with molecular masses ranging between 500 - 1,000,000 Kda are now available and have been applied to cereal proteins. Bietz (1984a), using a TSK 3000 SW column resolved a total gliadin extract into 5 major peaks, corresponding to molecular masses of 11,000, 25,000. 41,000, 63,000 and 65,000. He identified these as salt soluble protein, α,β-gliadins, γ-gliadins, ω-gliadins and HMM-gliadins respectively and proposed that the profiles were suitable for wheat varietal identification. Huebner and Bietz (1985) using HPLC of SDS solubilised glutenins, correlated the presence of high molecular mass protein on the HPLC pattern with good baking quality in the flour. Kruger and Marchylo (1985b) used the same technique to monitor the progress of germinating wheat seeds, showing that the content of HMM protein decreased and LMM protein increased as germination progressed.

1.3.4.3. Reversed-Phase High Performance Liquid Chromatography

Of the various HPLC techniques applied to proteins, RP-HPLC has been most widely used to fractionate and characterise cereal proteins, especially wheat proteins. Initial studies revealed that gliadin RP-HPLC profiles could be used for the variety identification of barley (Marchylo and Kruger, 1984; 1985; Wingad *et al.*, 1985; 1986) wheat (Bietz *et al.*, 1984b; Huebner and Bietz, 1985; 1987; Marchylo *et al.*, 1988b; Scanlon *et al.*, 1989a; 1989b), rice (Lookhart

et al., 1987), oats (Lookhart, 1985) and maize (Bietz, 1985b; Paulis and Bietz 1986).

Using gliadin RP-HPLC profiles of Chinese spring hexaploid wheat and its nullisomic, tetrasomic and hexaploid lines, Bietz and Burnouf (1985) and Burnouf and Bietz (1984a) confirmed electrophoretic results indicating that gliadins are controlled by genes on the short arms of the group 1 and group 6 chromosomes (the complex Gli-1, Gli-2 loci). This was determined in part by observing the following: ω-gliadins in early eluting peaks were coded by genes on the short arms of chromosomes 1A, 1B and 1D (Gli-1 loci); the α-/β-gliadins (in peaks of intermediate hydrophobicity) were coded by genes on the short arms of chromosomes 6A, 6B, 6D, (Gli-2 loci); the γ-gliadins (in late eluting hydrophobic peaks) were controlled by genes on the short arms of chromosomes 1A, 1B, 1D, (the gli-1 loci).

Gliadin RP-HPLC has been shown to complement electrophoresis as a method to determine the viscoelasticity of Durum wheats (Burnouf and Bietz, 1984a). More recently its application in predicting breadmaking quality in wheat has been explored. Huebner and Bietz (1986) showed that a positive correlation exists between the presence of particular gliadin peaks and good breadmaking quality in wheat. Lookhart and Albers (1988) identified an ω-gliadin found only in good quality wheat and a β-gliadin found only in poor quality wheat using both RP-HPLC and acid PAGE.

Glutenins now known to be closely associated with breadmaking quality have been separated by RP-HPLC. Burnouf and Bietz (1984b) found that HMM glutenin subunits elute

first when separated by RP-HPLC. Later Huebner and Bietz (1985) showed that relative amounts of HMM and LMM glutenin subunits, determined by quantitative RP-HPLC can be used to predict the "general breadmaking score", an indicator of breadmaking quality. Kruger et al. (1988) showed that the relative extractability in 50% v/v aqueous propanol, with or without a reducing agent, of HMM glutenins subunits was related to dough strength. It was subsequently shown that the RP-HPLC separation profile of the extracted protein could be used for quality prediction in bread wheats.

RP-HPLC was also applied to salt soluble proteins, extracted from wheat flour. More than 100 peaks were separated using a linear gradient from 15-51% v/v aqueous acetonitrile, at elevated temperatures. However the patterns were not found to be very reproducible (Bietz, 1983).

1.3.5. Advantages of High Performance Liquid Chromatography

i) Speed

For optimal resolution, RP-HPLC of gliadins requires 1-2 h. However at higher temperatures (70^{o}C) 10-15 min are usually adequate (Bietz, 1983; 1986; Bietz and Cobb, 1985). For rapid screening of Durum wheat varieties for pasta quality, gradients of about 5 min may reveal the desired sample characteristics (Burnouf and Bietz, 1984a). Run times for SE-HPLC of glutenins are usually only 20-30 min (Bietz, 1984a) and for IE-HPLC of gliadins run times of 15-20 min have been reported (Batey, 1984). In contrast, run times for comparable separations by gel filtrations, ion exchange chromatography, or hydrophobic interaction chromatography by

conventional means, frequently take days, rather than hours or min.

ii) Sensitivity

Because of the small efficient columns and the ability (with many solvents) to monitor proteins at 210 ηm, cereal protein HPLC requires very small samples. Bietz (1983; 1984b; 1985a; 1986) uses as little as 50ηg of protein per peak. This high sensitivity permits, analysis to be made from a half kernel of grain, while the germ may be saved for further evaluation.

iii) Resolution

In gliadin RP-HPLC, 40-100 peaks commonly resolve in about 1h (Bietz, 1983; 1984b; 1985a; 1986), thus exceeding the resolution obtained using conventional HIC (Popineau and Godon, 1978; Caldwell, 1979; Chung and Pomeranz, 1979). In addition the resolution generally exceeds that of 1 dimensional electrophoresis (1D) and approaches that of many 2D electrophoresis systems. Resolution of SE-HPLC (Bietz, 1984a) is similar to that obtained by conventional means and that of IE-HPLC of hordein proteins is similar to that of ID electrophoresis (Wingad et al., 1986).

iv) Reproducibility

Excellent reproducibility can be achieved using HPLC, primarily because the solvent delivery system and controllers permit accurate and constant flow rates and gradients. Further modifications to improve reproducibility include, maintaining constant column temperatures and deaeration of all solvents (Bietz and Cobb, 1985) before and during use.

v) Recovery

Bietz (1983) found by comparing the absorbance of applied and recovered samples that 96-98 percent of applied proteins were recovered in RP-HPLC. Other studies of non-cereal proteins report recoveries between 80-100 percent for proteins and large peptides from RP-HPLC columns (Regnier and Gooding, 1980; Hearn et al., 1982).

vi) Quantitation

One of the greatest advantages of HPLC is that data are easily quantified, generally more accurately than in conventional chromotography. Constant flow rates, rapid analyses and reliable injection and integration combined with the ability to monitor column effluent at 210 ηm are possible reasons for this capability (Bietz, 1985a).

vii) Automation

The availability of system controllers and automatic sample injectors in modern HPLC systems greatly increases the number of samples that may be analysed, by night or weekend operation. Automation also reduces the possibility of operator error. In addition reproducibility also improves because of the constant re-equilibration conditions for each sample and the application of accurate and reproducable sample injection volumes (Marchylo and Kruger, 1988a).

1.3.6. Limitations of High Performance Liquid Chromatography

The main limitation to the use of HPLC is its cost, due to the use of mechanically and electronically complex equipment. For cereal protein analysis, procedures

permitting interlaboratory comparisons of data, though the use of internal standards are not yet available. However HPLC is still evolving and is not meant to replace any existing technique, however its very complementary nature makes it a valuable addition to existing methods.

1.4. IMMUNOCHEMICAL ASPECTS OF CEREAL PROTEINS

The basic antigen-antibody reaction provides a means for very sensitive and specific analytical detection procedures. Since the first reports, about 58 years ago, on the use of antibodies for quantitative analytical purposes (Heidelberger and Kendall, 1932), immunology has been increasingly recognised as a valuable analytical tool. The origin and also the main application of immunological methods has been in the field of medicine but their use has now spread to many other disciplines including cereal chemistry (Vaag and Munck, 1986).

Polyclonal and monoclonal antibodies have been used to study the close homology of wheat storage protein components. However the low solubility and low antigenicity of these proteins has limited the use of antibodies. An additional problem arises from the variation in the response of the individual animals and furthermore some unimmunised rats and rabbits have been found to have indigenous antigliadin antibodies (Coombs et al., 1983).

Immunochemical studies (Fulchuck, 1974) on wheat protein revealed that wheat gliadin, especially α-gliadin is responsible for causing mucosol injury to the small intestine (coeliac disease). This prompted the development of immunoassays using polyclonal antisera for gliadin detection in "gluten free" food, prescribed for such patients, (Windemann et al., 1982; Ciclitera and Lennox, 1983; Troncone et al., 1986; Ayob et al., 1988). More recently immunoassays based on monoclonal antibodies have also been used (Mills et al., 1989).

1.4.1. Polyclonal Antisera

Early work on the immunochemistry of wheat storage proteins suggested that there were some common antigenic determinents among gliadins and glutenins (Benhamou et al., 1965); Grabar et al., 1965). This observation was disputed by Elton and Ewart (1963) and Ewart (1966) who suggested that contamination of the gliadin used, with glutenin residues may have been responsible for the reaction of the glutenin fraction with anti-gliadin antibodies. Cleavage of the disulphide bonds of gliadins abolished its ability to react and under the same conditions, glutenin lost its original antigenic structure and aquired a new one (Escribano et al., 1966; Escribano, 1967). Native gliadin produced a multiplicity of immunoprecipation lines (Elton and Ewart, 1963; Ewart, 1966; Nimmo and O'Sullivan, 1967) which indicated that gliadin fractions contained several immunologically distinct proteins. α-,β- and γ-gliadins were distinguished on the basis of their immunological reactions, however ω-gliadin gave no immunoprecipitation lines with antibodies raised to total gliadins (Booth and Ewart, 1970). It was also reported that the antigenic determinants of both α- and β-gliadins were missing on γ-gliadins (Beckwith et al., 1966). Vu and Popineau (1987) studied the immunochemical properties of γ44 and γ46 from common wheat and showed that these proteins have common antigenic determinants. Furthermore antisera raised against purified γ44 and γ46 did not react with other γ-gliadins or with α,-β,-ω-gliadins. Thus it appears that the antigenic determinants of γ-gliadin components correspond to

polypeptide structures that do not exist in other gliadins.

Studies with polyclonal antisera have shown immunological cross-reactivity between the prolamins from different cereals. Elton and Ewart (1963) raised antibodies in rabbits to bread wheat gluten and found this antiserum bound strongly to Durum wheat and rye prolamins but less strongly to barley. Rabbit anti-hordein antisera bound some wheat γ-and ω-gliadins (Dierks-Ventling, 1982). Marked species differences between the salt soluble proteins of hexaploid wheat and Durum wheat have been found as antibodies raised to a salt soluble protein fraction of Triticum aestiuum did not bind Durum wheat proteins, (Piazzi and Cantagalli, 1969; Piazzi et al., 1972).

Several immunoassays for gliadins have been developed, these include RIA's (Ciclitera and Lennox, 1983; Ciclitera et al., 1985) and enzymeimmunoassays (EIA) introducing both sandwich (Lane et al., 1982; Windemann et al., 1982; McKillop et al., 1985; Troncone et al., 1986; Ayob et al., 1988) and competitive (Windemann et al., 1982; McKillop et al., 1985) types. These assays are capable of detecting α-,β-,γ-gliadins and one is specific for α-gliadins (Ciclitera and Lennox, 1983) potentially the most toxic gliadin. One report (Meier et al., 1984) shows that in vitro gliadin toxicity is reduced by heating to 80^{o}C. None of these reports considers in any detail the effect of the sample matrix on the determination of residual gliadin in foods. However they all report cross-reactivity between anti-gliadin immune serum and other cereal prolamins (Gosling et al., 1985).

1.4.2 Monoclonal Antisera

The reactivity of monoclonal antibodies directed against common wheat endosperm protein shows considerable variation (Ullrich et al., 1986; Skerritt et al., 1984; 1986; Festenstein et al., 1987; Freedman et al., 1988). Skerritt et al. (1984) identified anti-gliadin clones with broad and narrow specficity. The former interacted with an extensive range of gliadin protein while the latter interacted with 6-10 discrete bands in the ω-,γ, group of proteins. Antibodies from either clone interacted with a pair of low mobility ω-gliadins. No clones were identified which reacted with only a single band and none were found to produce antibodies to higher mobility α-,β-gliadins. Sera from unimmunised mice also had anti-gliadin antibodies, although the titres for antibody detection were lower than those for sera of immunised mice. It has been suggested that this is a consequence of dietary absorbtion of gliadin peptides (Hemmings et al., 1976). All the antibodies tested were found to bind proteins from common wheat, Durum wheat, rye, rye grass and barley, only limited cross reactivity was found with rice.

In a similar experiment Freedman et al. (1988) raised monoclonal antibodies in rats and mice to gliadins from the wheat variety Recktor and showed identical cross-reactivity between gliadins separated by 1D electrophoresis and gliadin fractions separated by RP-HPLC. Antibodies bound strongly to α/β gliadins, γ-gliadins and to a lesser extent to unfractionated glutenin. A poor response was found to ω-gliadin, wheat albumin and globulin. The antibodies reacted

with prolamins from rye, barley, oats but not maize.

Theobald et al. (1983), produced a monoclonal antibody to a saline protein extract of flour and claimed it cross-reacts with the gliadins reported to be associated with pasta-making quality in Durum wheat (du Cros et al., 1982).

Immunoassays based on monoclonal antibodies have been used to detect the presence of gliadins in 'gluten free' foods. Skerritt (1985a), using nitrocellulose discs, developed a monoclonal antibody-based immunoassay to quantify "gliadin-like-immunoreactivity" in food. Freedman et al. (1987), and Mills et al. (1989), reported sandwich ELISAs using murine monoclonal antibodies prepared against unfractionated wheat gliadin. Dawood et al. (1989) showed that monoclonal antibodies have a potential application in variety identification of cereal cultivars. More recently a library of monoclonal antibodies have been prepared to wheat gluten protein by Mills et al. (1990), Skerritt and Lew (1990) and Skerritt and Hill (1990). These workers have confirmed that monoclonal antibodies cross-react with the grain storage proteins of related cereal species. Furthermore Skerritt and Hill (1990) showed that different cross-reactivity was found when the protein was extracted using different extractants and that nitrocellulose membranes were better solid phase supports than polystyrene microwells. Different cross-reactivity results were also obtained when the same grain protein extracts were used in indirect, antigen competition and sandwich-type assay formats.

1.4.3. Conclusions

The suggestion to use immunological methods to

detect cereal proteins in flour or foods is by no means new. Antibodies raised against wheat globulin (Piazzi and Cantagalli, 1969) and wheat albumin (Piazzi et al., 1972) have been used to assay for common wheat impurities in Durum wheat. Methods based on immunoturbidity of solutions or immunoprecipitation in gels using polyclonal antisera to gliadin have been reported (Keyser and Mahler, 1973; Baudner, 1977; Gombocz et al., 1981), these appear only to have applications to uncooked preparations such as flours or baking mixes. However the use of polyclonal and monoclonal antibodies with specificity for heat stable gluten proteins should allow the analysis of cooked and processed foods.

1.5. AIM OF THIS STUDY

Lactate PAGE of gliadins and gliadin RP-HPLC represent two complimentary approaches for the discrimination and identification of hexaploid and tetraploid wheat varieties (Bietz et al., 1984b). Worldwide, Canada, (Kruger and Marchylo, 1985a; Scanlon et al., 1989a; 1989b) USA, (Bietz et al., 1984b; Bietz and Cobb, 1985) Australia, (Lookhart and Pomeranz 1985) New Zealand (Cressey, 1987) and France (Burnouf et al., 1983) RP-HPLC has been proposed as an alternative to lactate PAGE for variety identification in wheat. The quantitative capabilities and automation potential (Scanlon et al., 1989b) of this technique are particularily advantageous especially for laboratories with a large sample through-put. An added advantage is the possible association between the elution of particular peaks in the RP-HPLC profile and the presence of particular technological properties in wheat (Burnouf and Bietz 1984a). The first aim of this study is to examine the potential of gliadin RP-HPLC profiles for varietal identification of UK wheat. Then, utilising its quantitative capabilities, its potential as a separation tool to replace lactate PAGE for the estimation of F1 hybrid purity will be explored. Hybrid purity is chiefly determined using lactate PAGE of single seed gliadin extracts in a sample of at least 100 seeds (Draper 1987). It would be advantageous to seed breeders and certification authorities if RP-HPLC could be applied successfully to estimate the purity from a batch of hybrid seeds.

Techniques such as the tlc separation of fatty acid sterols and the electrophoretic separation of either

cytoplasmic or gliadin proteins, have been proposed for the detection of common wheat adulteration of Durum wheat. However most workers agree that results obtained when these methods are applied to pasta products are very subjective and their ability to detect adulteration in pasta, processed at high temperatures is considerably reduced. A quantitative method capable of detecting low levels (<5%) of common wheat in pastas processed at elevated temperatures is optimally required.

RP-HPLC techniques, similar to those used for wheat variety identification tend to be more powerfully resolving than electrophoresis. The second aim of this project is to explore gliadin RP-HPLC profiles for the possibility of detecting a peak containing a protein unique to hexaploid wheat gliadin, the presence of which in Durum wheat or pasta gliadin extracts would serve as an indicator of common wheat adulteration. Using the integration capabilities of the RP-HPLC system the peak can be quantified and thereby related to the extent of adulteration.

Furthermore using the preparative capabilities of the reversed-phase system it may be possible to isolate sufficient amounts of protein to identify and characterise the protein. Purified protein can be used to immunise a suitable host animal. After collection of antisera developed immunochemical procedures can be used to isolate antibodies with specificity only towards common wheat gliadins. This antibody preparation can then be assessed for its ability to detect common wheat adulteration of pasta products.

2. MATERIALS AND METHODS

2.1. MATERIALS

All work pertaining to this study with the exception of the two experiments listed below was carried out in the laboratories of the department of Life Sciences, Nottingham Polytechnic. The tlc separation of fatty acid sterols was carried out by the Food and Bioscience division at the Laboratory of the Government Chemist (LGC), Teddington Middlesex, UK. The determination of purity in F1 hybrid wheat using acid PAGE of gliadins extracted from individual wheat grains was carried out at the National Institute of Agricultural Botany, Cambridge, UK.

2.1.1. Source of Chemicals

Chemicals used for electrophoresis and isoelectric focussing were of Electran grade, those used for buffers and immunochemical determinations were of AnalaR grade, all other chemicals were general purpose reagent grade. All organic solvents used in conjunction with RP-HPLC were of HiPerSolv grade. The following list identifies the source of chemicals.

i) Organic solvents for RP-HPLC

Acetonitrile; trifluoroacetic acid (SpectrosoL); methanol were obtained from British Drug Houses, Poole, Dorset, UK.

ii) Chemicals for electrophoresis

Acrylamide; N,N'-methylenebisacrylamide; glycine; trichloroacetic acid; urea; ferrous sulphate; ascorbic acid; sodium dodecylsulphate; N,N,N',N'-tetramethylenediamine; ammonium persulphate; glycerol; bromophenol blue; coomassie brilliant blue R-250; boric acid; 2-dimethylaminoethyl

cyanide; sodium phosphate; acetic acid; dimethylformamide; ethylenediaminetetraacetic acid; acetone; ethanol were obtained from British Drug Houses, Poole, Dorset, UK. Hydrogen peroxide; pyronine Y;αnaphtyl acetate; lactic acid; tris(hydroxymethyl)aminomethane; fast blue RR salt; protein molecular weight standards were obtained from the Sigma Chemical Co., Poole, Dorset, UK. Catechol and 2-chloroethanol were obtained from the Aldrich Chemical Co., Gillingham, Dorset, UK. Ampholine PAG plates pH range 3.5-9.5, carrier ampholine solution pH range 3.5-9.5 and Repel Silane (dimethylchlorosilane 2% w/v in 1,1,1 trichloroethane) were obtained from LKB-Producter AB, Bromma, Sweden and aluminium lactate was obtained from May and Baker, Manchester, UK.

iii) Chemicals for thin layer chromatography

Petroleum ether; ethyl ether; carbon tetrachloride were obtained from British Drug Houses, Poole, Dorset, UK. Phosphomolybdic acid was obtained from FSA laboratories, Loughborough, UK and tlc aluminium sheets coated with silica gel (0.2mm) from Merck, Darmstadt, FRG.

iv) Chemicals for immunochemistry

Freunds complete adjuvant; Freunds incomplete adjuvant; sodium acetate, sodium azide, polyoxyethylene sorbitan monolaurate; ponceau S; poly-L-lysine; 4-chloro-1-naphthol; 3,3,5,5, tetramethylbenzidene; cyanogen bromide sepharose 4B; antibodies (including anti-rabbit HRP, anti-rabbit IgG biotin conjugate, peroxidase labelled avidin); o-phenylene diamine dihydrochloride were obtained from the Sigma Chemical Co., Poole, Dorset, UK. Ethanolamine;

dimethylsulphoxide; glutaraldehyde; 1,4 dioxane; sodium chloride; potassium chloride; sodium phosphate; dibasic sodium phosphate; dibasic potassium phosphate were obtained from British Drug Houses, Poole, Dorset, UK.

2.1.1.1. Miscellaneous Items

Elisa plates (96 well microtest) were obtained from Becton Dickinson Labware, Oxnard, California, USA; electrode wicks were obtained from Whatman Labsales Ltd., Maidstone, Kent, UK; guard columns were obtained from Technicol Ltd., Stockport, Cheshire, UK; marvel dried milk was obtained from Cadbury Premier brand (UK), Bournville, Birmingham, UK; nitrocellulose paper and Nylaflo filters 0.45μm pore size were obtained from Gelman Sciences Inc, Ann Arbor, Mississippi, USA; nitrocellulose filters, 0.22μm and 0.8μm pore size were obtained from from Waters Millipore, Middlesex, UK; Rabbit anaesthetics: Hypnorm was obtained from Janssen Pharmaceutical Ltd., Oxford, UK and Hypnovel was obtained from Roche Products Ltd., Welwyn Garden City, UK.

2.1.2. Laboratory Equipment

The following list identifies the laboratory equipment used:

Centrifuges:

Centrifuge	Rotor
MSE Microcentaur (swing-out)	12 x 1.5 ml
MSE 21	8 x 50 ml (angle)

RCF (x g_{av}) values were calculated from nomogram tables supplied by the manufacturers MSE Scientific instruments, Crawley, Sussex, UK.

Electrophoresis equipment

Vertical electrophoresis - LKB 2001 electrophoresis unit

Horizontal electrophoresis - LKB 2117 - 001 Multiphor 11

Isoelectric focussing - LKB 2117 - 003 Electfocussing unit

Electrophoretic transfer - LKB 2117 - 005 Multiphor 11

were all obtained from LKB - Produkter AB, Bromma, Sweden.

Protean II vertical electrophoresis system was obtained from BIO-RAD, Richmond, California, USA.

End over end rotator (TAAB rotator type N) was obtained from Gallenkamp, Loughborough, UK.

Gallenkamp flask shaker was obtained from Gallenkamp, Loughborough, UK.

High-performance liquid chromotography equipment consisted of a Waters Associates Model 6000A, a Waters Millipore Model 510 solvent delivery systems and a Millipore Waters Model 680 automated gradient controller obtained from Millipore Waters, Middlesex, UK. Samples were injected with an LKB 2157 autosampler and monitored in an LKB 2238 Uvicord S 11 single beam UV spectrophotometer. Fractions were collected in an LKB 2211 Superfrac fraction collector obtained from LKB Produkter AB, Bromma, Sweden. A Nelson Analytical 900 series interface box (intergrater software serial number 00001418) was obtained from Nelson Analytical, Cupertino, California, USA and a personal computer from Walters International, High Wycombe, UK, was used for aquiring and processing liquid chromatography data. A Tecam C-100 water circulator from Techne, Cambridge, UK, was used to circulate water through a glass jacket surrounding the chromatography column.

Laboratory mill (Moulinex coffee bean grinder): was supplied

by a local store and manufactured by Moulinex, France.

pH meter: Pye Unicam PW 9401 was supplied by Pye Unicam, Cambridge, UK; Beckman 0 71 supplied by Beckamn Instruments, Palo Alto, California, USA.

Titertek multiscan plus ELISA reader was supplied by Flow Laboratories, Rickmansworth, Herts., UK.

2.1.3. Source of Wheat and Pasta

Seeds of European bred Durum wheat cultivars (Durum Flodur, Capdur, Arcour, Valdur, Casour, Regal, Darius, Durelle, Neodur, Primadur, Ardente, Mondur, Decius, Ambral) and European bred common wheat culivars (Avalon, Maris Dove, Crossbow, Rapier, Monza, Pernel, Sabre, Bounty, Baron, Mission, Norman, Maris Huntsman, Alexandria, Brigand, Broom, Brimstone, Ferman, Jerico, Minaret, Kanzler, Hustler, Brock, Galahad, Hobbit, Mercia, Rendezvous, Solitare, Moulin, Hornet, Stetson, Slejpner, Parade, Riband, Tonic, Axona, Apollo, Pastiche, Wembley) were obtained from authenticated stock held at the National Institute of Agricultural Botany, Cambridge, UK and the Plant Physiology section, Nottingham Polytechnic, Nottingham, UK. North American bred Durum wheat cultivars (Vic, Lloyd, Ward, Edmore, Benville, Quilafen) were received from The College of Agriculture, North Dakota State University, Fargo, North Dakota, USA. Two mixtures (French, Belgian and English Durum) of 100% Durum semolina and samples of pasta suspected of containing common wheat adulteration were received from the LGC, Teddington, Middlesex, UK. Commercial samples of Durum wheat, semolina flour and pasta were obtained from Pasta Foods, Great Yarmouth, Norfolk, UK.

2.1.3.1. F1 Hybrid Wheat

All wheat samples for F1 hybrid purity analysis were obtained from the National Institute of Agricultural Botany (NIAB), Cambridge, UK. Names of F1 hybrid wheat and F1 hybrid parent varieties are withheld for reasons of confidentiality. The production of F1 hybrid seed of S♂ x S♀, was carried out under the supervision of the NIAB.

2.1.3.2. Commercially Manufactured Pasta

Commercial samples of Durum wheat, semolina flour and pasta dried at "normal" and "superheat" conditions, were a gift from Pasta Foods Ltd, (Great Yarmouth, Norfolk, UK). Throughout the text the commercial pasta products will be referred to as low temperature pasta and high temperature pasta respectively. Low temperature pasta was extruded at 45°C and dried at 40°C for 22h, high temperature pasta was extruded at 45°C and dried at 70°C for 4h. Pasta, suspected of containing commom wheat was received from the (LGC).

2.1.3.3. Manufacture of Laboratory Pasta

Semolina flour and common wheat flour (from Smiths Mills, Worksop, Nottinghamshire, UK) were used to manufacture pastas adulterated with 0%, 5%, 10%, 20% and 30% common wheat using a Brabender single screw laboratory extruder. Extrusion conditions were as follows: temperature, 50°C; barrel speed, 95 rpm; and torque of 2. Pasta was dried in a laboratory drying oven at 40°C for 22h. Two pastas, one manufactured from 100% Durum wheat and another suspected of containing common wheat were rehydrated in tap water at 4°C, and then redried at 85°C for 4h. Samples of these were reassayed using each analytical technique.

2.2 ANIMALS

Polyclonal antibodies were raised in New Zealand white rabbits (both sexes) supplied by Shrubacre Rabbits, Beccles, Suffolk, UK. Rabbits were housed in aluminium cages (45x60x79cm) and kept in an air conditioned animal room with a temperature between 16-18^{o}C.

2.3. EXTRACTION PROCEDURES FOR WHEAT PROTEINS AND STEROLS

2.3.1. Gliadin Extraction

For F1 hybrid purity analysis using RP-HPLC either a single seed was ground to a flour and the gliadins extracted with 0.5ml of 70% (v/v) aqueous ethanol or a batch sample of seeds (100 seeds) was ground to a flour and the gliadins extracted with 20ml of 70% (v/v) aqueous ethanol. Extractions were carried out at room temperature for 30 min or overnight at $4^{o}C$, after which the suspension was mixed for two min on a flask shaker the supernatant was separated by centrifugation (12,000g x 10 min). Protein content was estimated according to the method outlined in section 2.7.

For detection of common wheat adulteration of Durum wheat or pasta using RP-HPLC, wheat flour was extracted with 25% (v/v) aqueous 2-chloroethanol (100 mg ml^{-1}), addition of solvent was followed by vigorous mixing for 2 min followed by standing in the cold room for 30 min. The mixture was then vigorously mixed for 1 min. Samples were clarified by centrigation (12,000g x 10 min) and filtered through a 0.45 μm pore size filter (Waters Millipore) before injection onto the column. Protein content was estimated according to the method outlined in section 2.7.

For acid polyacrylamide gel electrophoresis (acid PAGE) gliadins were extracted from samples of milled wheat with 25% (v/v) aqueous 2-chloroethanol (250 mg ml^{-1}) in the cold room for at least 2h, or, more usually overnight.

For immunochemical studies of gliadins the following extracts were prepared:

i) Total Gliadin Extract of Avalon

Freshly milled wheat (2g) was mixed with 20ml of 70% (v/v) aqueous ethanol and extracted with constant stirring for 2h at room temperature. The mixture was transferred into a 50ml centrifuge tube and centrifuged at 10,000 x g for 20 min. The supernatant was retained and filtered through a 0.45μm pore size filter, transferred into a dialysis bag and dialysed overnight against 0.01M acetic acid with two changes of buffer. The dialysate was then collected and centrifuged at 10,000 x g before freeze drying.

ii) RP-HPLC Fractionated Gliadins

Fractions eluting between 47-49 min on the RP-HPLC profiles of a total extract of Avalon gliadins were collected and freeze dried. Numerous separations were carried out until 10mg of protein was separated. After acid electrophoresis to identify the proteins (γ-/β-gliadins) the semi-pure protein was dialysed overnight against 2 x 1L of 10mM acetic acid and freeze dried. This was stored at -20^{o}C until required for the immunisation of rabbits.

2.3.2. Extraction of Polyphenoloxidases

Polyphenoloxidases were extracted in 1M urea from wheat and pasta samples (100mg ml^{-1}) by gentle shaking at room temperature for 30 min. After centrifugation (12,000xg) the supernatant was collected and retained for alkaline PAGE.

2.3.3. Extraction of Esterases

Esterases were extracted in distilled water from wheat and pasta samples (100mg ml^{-1}) by gentle shaking at room temperature for 30 min. After centrifugation (12,000xg) the supernatant was retained for isoelectric focussing.

2.3.4. Extraction of Wheat Sterols

Wheat or pasta (20g) was ground using a coffee mill to pass through a seive with a 0.2mm mesh size. The sample was mixed with 150ml of petroleum ether and after standing at room temperature overnight the mixture was filtered on a Buchner funnel fitted with a sintered glass filter. The clear solution obtained was transferred into a pre-weighed 100ml calibrated flask and the solvent was evaporated under reduced pressure by heating the flask in a waterbath at 40-50^{o}C. When evaporation was complete the flask and contents were heated under reduced pressure for a further 10 min. The flask was dried and allowed to cool. The weight of extract was determined and diluted in ethyl ether on the basis of 1ml of ethyl ether per 60mg of extract.

2.4 POLYACRYLAMIDE GEL ELECTROPHORETIC METHODS

2.4.1. Acid Gel Electrophoresis of Wheat Gliadins

The method used was that of Draper (1987) using the vertical slab gel apparatus which is widely used for separating proteins.

2.4.1.1 Sample Preparation

Gliadin extracts containing 2mg protein ml^{-1}, in 25% (v/v) aqueous 2-chloroethanol (section 2.3.1.) were loaded directly into sample wells on the gel.

2.4.1.2. Solutions

The following solutions were made up in double distilled water:

Electrode Buffer (67mM acetic acid 5mM glycine)	4ml glacial acetic acid 0.4g glycine in 1L pH 3.1
Acrylamide Stock Solution (33.5mM acetic acid 13mM glycine)	5ml glacial acetic acid 250mg glycine 15g urea 250mg ascorbic acid 10mg ferris sulphate 25g acrylamide 1.25 g N'-N-methylene-bisacrylamide in 250ml pH 3.2
Sample Buffer	25ml 2-chloroethanol 50mg pyronine Y in 100 ml
Hydrogen Peroxide (0.6% v/v)	0.1 ml of 30% hydrogen peroxide in 5ml

2.4.1.3. Gel preparation and Electrophroresis

The LKB gel mould apparatus for vertical gels was assembled to produce 1.5mm thick gels according to the design of the equipment. The glass plates (13 x 16 cm) were thoroughly cleaned with detergent followed by distilled water and filtered ethanol and then silanated with Repel Silane as urea gels have a tendancy to stick to untreated glass. This facilitates easy removal of the gel after electrophoresis. Prior to pouring the gel the acrylamide stock solution (50 ml) was transferred to a clean Buchner flask, degased for 10 min and then cooled down to 4°C. Immediately after the addition of 150µl of freshly diluted 0.6% (v/v) aq hydrogen peroxide the solution was mixed and the gel was poured. To ensure satisfactory polymerisation of the upper gel surface the mixture was poured to overfill the top of the gel mould which contained an acrylic comb for the formation of samples wells. Polymerisation of the gel mixture occurs within a few minutes of adding the peroxide catalyst solution but 1.5h is allowed before the comb is carefully removed and the sample wells washed with electrode buffer.

These gels had the following compositions:

acrylamide	10% (w/v)
N',N methylenebisacrylamide	0.5% (w/v)
Urea	6% (w/v)
Acetic Acid	33.5 mM
Glycine	13 mM

The rest of the electrophoresis apparatus was then assembled and an appropriate volume of tank buffer was added

to the upper and lower buffer chambers. Subsequently pre-electrophoresis was carried out for 30 min at 400V and at 16°C with the electrode connections attached in the reverse polarity mode. Pre-electrophoresis helps to remove any charged impurities in the gel which could cause band distortion. Samples (5-10 µl of extract equivalent to 10-20 µg of protein) were then loaded into the bottom of the sample wells using a microlitre syringe. Electrophoresis was carried out at 400V for twice the time required for the pyronine Y marker to leave the gel, which for this system was usually about 3h.

2.4.1.4. Fixing Staining and Destaining of Gels

Following electrophoresis the gel was carefully transferred from the mould to a clean glass dish. Gels were fixed and stained in a solution of 0.02% (w/v) aqueous coomassie brilliant blue R-250 in 5% (v/v) aq ethanol, 12% (v/v) aqueous TCA (the dye was dissolved first in 95% (v/v) aqueous ethanol, filtered and then added to the TCA solution). Gels were usually stained overnight and destained in tap water for 1h before photography. For storage, gels were placed in polythene bags at 4°C.

2.4.1.5. Determination of Rm Values of Gliadins Separated by Acid PAGE

In the first dimension the Rm values of electrophoretically separated gliadin proteins were determined using the method of Sapirstein and Bushuk (1985). This nomenclature uses a gliadin band (first identified on the Canadian cultivar Marquis) present on the electrophoregrams of all wheat varieties as a reference to

which an arbitrary mobility of 0.50 (Figure 3.26.) is assigned. All other bands are identified on the basis of their electrophoretic mobility relative to 0.50 and are expressed in terms of the relative mobility X 100.

2.4.2. Gel electrophoresis of Wheat polyphenoloxidases

The method of Feillet and Kobrehel (1974) was used.

2.4.2.1. Sample Preparation

Polyphenoloxidase extracts containing 2mg protein ml^{-1}, in 6M urea (section 2.3.2.) were loaded directly into sample wells on the gel.

2.4.2.2. Solutions

The following solutions were made up in double distilled water:

Electrode Buffer	6.05 g tris
(50 mM Tris)	40.50 ml 0.2 M HCl in 1L
	pH 8.6
Acrylamide Stock Solution	8 g acrylamide
pH 8.6	0.4 g N',N
	methylenebisacrylamide in
	100ml of electrode buffer
	pH 8.6
Sample Buffer	6 g urea
(1 M urea)	50 mg pyronine y in 100ml
Catechol Staining Solution	1.95 g Tris
(16 mM Tris 0.7 mM EDTA)	2.50 g catechol
pH 7.5	0.20 g EDTA
	0.15 g boric acid in 1L
	pH 7.5

2.4.2.3. Gel Preparation and Electrophoresis

The LKB gel mould apparatus for vertical gels was assembled to produce 2.5mm gels as outlined in section 2.4.1.3. The acrylamide stock solution (100ml) was transferred to a clean Buchner flask, degased for 10min and after mixing in 350μl of DMAPN and 0.12g ammonium persulphate the gel was poured to overfill the top of the gel mould which contained an acrylic comb for the formation of sample wells. After polymerisation (approx 30 min) the comb was removed, the sample wells washed with electrode buffer and the remainder of the electrophoresis apparatus assembled. The gel was then allowed equilibrate overnight against electrode buffer.

These gels had the following compositions:

Acrylamide	8% (w/v)
N',N methylenebisacrylamide	0.4% (w/v)
Tris	50 mM

Pre-electrophoresis was carried out at 250V for 1h, with the electrode connections attached in reverse. Following this, 20μl of 6% (w/v) urea extract was loaded into the bottom of each well and electrophoresis was carried out at a constant temperature of 16^{o}C for 4h at 400V.

2.4.2.4. Staining for Polyphenoloxidases Activity

Following electrophoresis the gel was carefully transferred from the mould and soaked in a 5% (w/v) aluminium lactate solution for 10 min, washed with water and placed in a glass tray containing 500ml of catechol staining solution. A more intense stain is obtained if this solution is prepared one day in advance. To aid staining, it is important to keep

the gels stationary during the staining period. Gels were usually stained overnight, no destaining was required before photography. Gels were stored indefinitely in 3% (v/v) aqueous acetic acid.

2.4.3. Gradient SDS-Polyacrylamide gel electrophoresis of Wheat Gliadins

The method employed was a modification of that described by Laemmli (1970).

2.4.3.1. Solutions

The following solutions were made up in double distilled water:

Tris-glycine electrode buffer	15.2g Tris base
(25mM Tris, 192mM glycine)	72.0g glycine
	5.0g SDS
	pH 8.5 in 5000ml
Tris-SDS stock solution	3.03g Tris base
(0.25M Tris) pH 6.8	0.2g SDS in 100ml
	pH 6.8
Tris-SDS stock solution	9.08g Tris base
(0.75M Tris) pH 8.8	0.2g SDS in 100ml
	pH 8.8
Sample buffer	25ml Tris-SDS stock pH
	pH 6.8
	2g SDS
	10ml glycerol
	0.1ml 1% bromophenol blue
	made up to 100ml
Acrylamide stock solution	30g acrylamide
	0.8g N'-N-methylene-

bisacrylamide to 100 ml

Ammonium persulphate 2% (w/v freshly prepared)

2.4.3.2. Sample Preparation

Total gliadin extracts (section 2.3.1.3.) containing 2mg ml^{-1} of protein were diluted 1:1 in sample buffer (62.5mM Tris, 2% (w/v) SDS, 10% (v/v) glycerol and 0.01mg/ml bromophenol blue pH 6.8). Freeze dried RP-HPLC fractions containing gliadins were first resolubilised in 25% (v/v) aqueous 2-chloroethanol (2mg ml^{-1}) and then diluted 1:1 in sample buffer. Prior to electrophoresis samples were incubated at 60°C for 10 min to ensure solubilisation of the protein.

2.4.3.3. Gel Preparation and Electrophoresis

The LKB gel mould apparatus for vertical slab gel electrophoresis was assembled as outlined in section 2.4.1.3. The gradient former (capacity, 2 x 15ml chambers), with a plastic tube attached from the outlet chamber (which contained a magnetic pellet) to a peristalsic pump was placed on a magnetic mixer. The plastic tubing leading from the pump was fitted between the glass plates to the bottom of the gel mould,

The following acrylamide solutions were prepared:

	Dense acrylamide (20% w/v)	Light acrylamide (10% w/v)
Acrylamide stock (30%)	20ml	10ml
Tris-SDS stock pH 8.8	5ml	5ml
Glycerol	5ml	-
Water	-	15ml

Solutions were transferred to Buchner flasks and degassed for 30 min. Immediately before pouring the gels

150μl of freshly prepared 1% (w/v) ammonium persulphate and 5μl of TEMED were added to each acrylamide solution and gently mixed. Equal volumes (14ml) of each solution were pipetted into separate chambers of the gradient former. At commencement of pumping (3ml min^{-1}) the tap connecting both chambers was opened allowing the light solution to enter the outlet chamber and mix with the heavy solution. Pouring was complete within 10 min and the gel was then overlaid with a layer of water saturated butan-2-ol, which excluded air and created a flat top to the gel. Polymerisation was allowed to proceed for 2h at room temperature after which the overlay of butan-2-ol was replaced by 50% (v/v) Tris-SDS stock pH 8.8. The gel was sealed with a layer of polythene and left to complete polymerisation overnight. Before setting the stacking gel, the overlay solution was poured off and any excess was carefully absorbed with filter paper.

The stacking gel solution was prepared by combining 2ml of acrylamide stock, 10ml Tris-SDS stock solution pH 8.8, and 8ml of distilled water. Following degassing for 10 min 0.5ml of freshly prepared 1% (w/v) ammonium persulphate solution and 7μl of TEMED were added and the solution thoroughly mixed. This was poured on top of the resolving gel and allowed to overfill the gel mould which contained an acrylic comb for the formation of sample wells. Polymerisation was allowed to proceed for at least one hour at room temperature. The comb was carefully removed and the wells washed 3-4 times with electrode buffer prior to being filled with electrode buffer ready for sample loading. Samples were added to the bottom of each well using a

microlitre syringe. The rest of the electrophoresis apparatus was assembled and electrophoresis was carried out at 16°C at a constant current of 20mA per gel for approximately 5h, until the tracking dye was 1 cm from the bottom of the gels. Standard proteins of known molecular weight were also electrophoresed beside the samples on the gel to allow calibration for determination of protein molecular weight. The protein standards used were Carbonic anhydrase (30,000), Egg albumin (45,000), Bovine Serum Albumin (67,000), phosphorylase b (94,000) and beta-galactosidase (116,000).

2.4.3.4. Fixing Staining and Destaining of Gels

After electrophoresis, the migration distance of the bromophenol blue dye front was recorded and the whole gel carefully transferred from the mould to a clean glass dish containing the fixing and staining solution (1.25g PAGE Blue 83, 18% (v/v) aqueous methanol and 5% (v/v) aqueous glacial acetic acid). Staining continued for 2h or overnight at room temperature after which destaining of gels was achieved by their immersion in 500ml of a solution containing 18% (v/v) aqueous methanol, 5% (v/v) aqueous glacial acetic acid. Several buffer changes were made until the gel was free of background colour. This process was hastened by gentle heating (37°C) and agitation of the solution. After photography fully destained gels were stored indefinitely in destaining solution.

2.4.4. Two Dimensional Electrophoresis

The method used was a modification of the method of Lafiandra and Kasarda (1985).

2.4.4.1. Sample Preparation

Total extracts (2mg protein ml^{-1}) of Avalon and D. Valdur gliadins were extracted as outlined in section 2.3.1.3. Fractions collected from the RP-HPLC separation of total gliadin extracts were freeze dried and resolublised in 25% (v/v) aqueous 2-chloroethanol before electrophoresis. Sample loadings of 8μl and 20μl were applied were applied for 1D electrophoresis and 2D electrophoresis respectively.

2.4.4.2. First-Dimension Electrophoresis

First dimension electrophoresis was carried out as outlined in section 2.4.1. using the Protean II vertical slab electrophoresis apparatus assembled to give gels 20 CM long.

2.4.4.3. Second-Dimension Electrophoresis

When a 2-D separation was to be carried out, a single sample was loaded into a central slot (or a slot displaced slightly from the centre, depending on the nature of the 2-D pattern) for the first dimension of electrophoresis. A similar sample was also loaded in an end slot: this end part of the gel was then cut off and stained to provide a 1-D reference pattern before the second-dimension separation was carried out.

After the first electrophoretic separation gels were removed from the plates and equilibrated with gentle shaking for 30-45 min with 1L of equilibration buffer (0.125M Tris, 0.025M glycine, 1.5M DMF, pH 9.2). DMF is important in maintaining the solubility of the wheat gliadin proteins.

The second-dimension electrophoresis was carried out using the LKB multiphor II horizontal electrophoresis

apparatus assembled according to the manufacturers instructions. A transparent cellulose acetate sheet was placed on the cooling plate of the apparatus and the gel positioned on the sheet so that the electric field would be applied at 90^{o} to the field of the first dimension. Strips of filter paper cut to gel size, were saturated with equilibration buffer contained in the buffer chambers and then laid over the edges of the gel to connect buffer and gel. Electrophoresis in the second dimension was then carried out for 4h at 20mA per gel.

2.4.4.4. Gel Staining and Destaining

The 1D gel electrophoresis strip was fixed and stained as outlined in section 2.4.1.4. however 2 dimensional gels were stained for 24h to ensure complete staining.

2.4.5 Gel Electrofocussing

2.4.5.1. Electrofocussing of Wheat Esterases

Electrofocussing of wheat esterases was carried out under the conditions described by Ainsworth et al. (1984).

2.4.5.1.1. Sample Preparation

Extracts of wheat esterases in distilled water (section 2.3.3.) were loaded directly on the gel.

2.4.5.1.2. Gel Preparation and Electrophoresis

Isoelectric focussing of wheat esterases was carried out on ampholine PAG plates (containing 5% acrylamide, 2.4% v/v ampholines) pH range 3.5-9.5 positioned on a multiphor II apparatus assembled to the manufacturers instructions. Electrode strips (2) were soaked in anode buffer (1M H_3PO_4) and cathode buffer (1M NaOH). Excess

solution was removed with a tissue and the strips were applied close to the long edges of the gel. A sharp scissors was used to cut off parts of the strips which protruded beyond the end of the gel. Dry sample application pieces were applied to the surface of the gel using the template screen printed on the cooling plate as a guide. Immediately before commencement of electrofoccusing aliquots of supernatant 20μl) containing wheat esterases were applied using a micro pipette. The rest of the apparatus was assembled according to the manufacturers instructions and electrofoccusing was carried out, with cooling to 16^{o}C at a constant voltage of 500V for 2h. Although it was possible to visualise distinct ampholine bands within 1h a second hour was required to allow complete separation and focussing of the protein.

2.4.5.1.3. Staining for Esterase Activity

IEF gels were stained overnight for esterase activity using a mixture of α-naphtyl acetate (50mg) and fast blue RR salt (100mg) dissolved in 5ml of acetone and made up to 100 ml with 0.05M phosphate buffer pH 7.5. If necessary gels were destained in 7% (v/v) aqueous acetic acid before photography and storage in 3% (v/v) aqueous acetic acid.

2.4.5.2. Electrofocussing of Wheat Gliadins

Electrofocussing of wheat gliadins was carried out using a modification of the method described by O'Farrell et al. (1975).

2.4.5.2.1. Solutions

The following solutions were made up in double distilled water:

Acrylamide stock solution	30g acrylamide 0.8g N'-N-methylene- bisacrylamide to 100 ml
Ammonium persulphate	1% (w/v freshly prepared)
Ampholines pH 3-10	Used as supplied (40%w/v)
Sample Buffer (2M urea)	12g urea in 100ml
Fixing Solution	300 ml methanol 100g TCA 35g sulfosalicyclic acid 5g cupric sulphate in 1L.
Washing Solution	300 ml methanol 120g TCA 5g cupric sulphate in 1L

2.4.5.2.2. Sample Preparation

Freeze dried 70% (v/v) ethanol extracts of wheat gliadins or RP-HPLC fractionated gliadins (2mg ml^{-1}) were resolubilised in sample buffer (diluted 1:1) and loaded directly on the gel.

2.4.5.2.3. Gel Preparation and Electrophoresis

The Bio-Rad Protean II gel mould apparatus for vertical gels was assembled to produce 1.0mm thick gels according to the design of the equipment. The glass plates

(13 x 20 cm) were thoroughly cleaned with detergent followed by distilled water and filtered ethanol and then silanated with Repel Silane to prevent the gel from sticking to the glass. The acrylamide solution was prepared by mixing 12.0 ml of acrylamide stock solution and 3.0 ml of carrier ampholine solution and 35 ml of double distilled water. This solution was transferred to a clean Buchner flask and degased for 30 min. Immediately before pouring the gel, 2.5ml of freshly made 1% (w/v) ammonium persulphate solution was added and the solution was thoroughly mixed. Two gels were poured to about 5mm from the top of the gel mould. The gel solution was overlayed with butan-2-ol to exclude air and create a flat surface to the gel. Polymerisation of the gel mixture occurs within 1h of adding the persulphate solution, a further 1.5h was allowed before the gel was used. These gels had the following compositions:

Acrylamide	8% (w/v)
Ampholines	2.4% (w/v)
Urea	6% (w/v)

After polymerisation the gels were removed from the glass plates and positioned on a clean glass plate on the multiphor II apparatus. The remaining apparatus was then assembled and electrode strips were applied to the gel. Pre-focussing was carried out using a constant voltage of 500V until the current dropped to 5mA. Samples (20 μl) were then applied as in section 2.4.5.1.2. and electrofocussing was carried out at 1000V for 4h.

2.4.5.2.4. Staining

After electrofocussing the gel was removed from

the multiphor apparatus. The protein was fixed by agitating the gel in fixing solution overnight followed by at least 2h in several volumes of washing solution to ensure complete removal of the carrier ampholines. Gels were stained in a solution of 0.02% (w/v) aqueous coomassie brilliant blue R-250 in 5% (v/v) aq ethanol, 12% (v/v) aqueous TCA (the dye was dissolved first in 95% (v/v) aqueous ethanol, filtered and then added to the TCA solution). Gels were usually stained overnight and destained in tap water for 1h before photography. For storage, gels were placed in polythene bags at $4^{o}C$.

2.5. THIN LAYER CHROMATOGRAPHY

Sterols esters extracted from wheat or pasta were separated by tlc according to the method of Gillies and Young (1964) as modified by Bernaerts and Gruner (1968).

2.5.1. Sample Preparation

Wheat sterol extracts in ethyl ether (section 2.3.4.) were used.

2.5.2. Preparation of TLC Plates

The tlc plates were activated by heating to 130°C for 3h and then allowed to cool in a desiccator containing silica gel. The sample (20μl) was applied drop-wise to form a band of constant width and 3cm in length and the solvent allowed to evaporate.

2.5.3. Development of TLC Plates

The chromatogram was developed at room temperature with carbon tetrachloride using a chromatographic container, the walls of which were covered with filter paper soaked in solvent. As soon as the solvent had migrated 18cm the plate was removed and the solvent allowed to evaporate in a fume hood. When dry the chromatogram was developed a second time and the solvent again allowed to evaporate. It was then sprayed with a solution of 20% (v/v) aqueous phosphomolybdic acid in ethanol. The plate was by then a uniform yellow colour. Bands were developed by heating the sprayed plate at 110°C for 5 min.

2.6. REVERSED-PHASE HPLC

All chromatography data were obtained using a Waters Associates model 6000A, and Waters Millipore model 510 solvent delivery systems and a Waters Millipore model 680 automated gradient controller. Samples were injected through a Rheodyne 7000 injection valve connected to an LKB 2157 autosampler and eluted proteins were monitored at 206ηm using an LKB 2238 Uvicord SII single beam UV monitor. Water was doubly distilled and purified using a Waters Millipore Milli-Q purification system to a conductivity of 18$\mu\omega$. A Tecam C-100 water circulator was used to circulate water at constant temperature to a glass column jacket in order to maintain constant column temperature during RP-HPLC. A Waters, Delta Pak, C18, 5μm particle size reverse phase column (150x3.9mm) was used when observation and quantification of differences was required and a larger column (300mmx4mm, Waters Delta Pak, C18, 10μm particle size) was used when eluting proteins were collected for freeze drying and storage. A nucleosil based, C18 cartridge guard column was installed between the injector and the column. After each analysis the column was washed with 100% solvent B over 5 min before returning to initial conditions and application of the next sample.

2.6.1. Sample Preparation

Gliadins were extracted as outlined in sections 2.3.1.1. and 2.3.1.2. Prior to injection samples were filtered through 0.45μm pore size filters. Protein estimations (section 2.7), were carried out on all gliadin extracts which were then diluted with 70% (v/v) aqueous ethanol to a protein concentration of 2mg/ml. Injections

(50μl) of the diluted sample equivalent to 100μg of protein were applied to the column.

2.6.2. Elution Conditions

The RP-HPLC solvent elution programme was a modification of that proposed by Bietz et al. (1984).

Solvent A*: 15% (v/v) aqueous acetonitrile + 0.1% TFA

Solvent B*: 80% (v/v) aqueous acetonitrile + 0.1% TFA

Elution Gradient: 30.0-47.5 acetonitrile

Run Time: 55 min

Flow Rate: 0.5ml min^{-1} using C18 5μm particle size column

1.0ml min^{-1} using C18 10μm particle size column

Temperature: analysis of hybrid wheat at 60^{o}C

analysis of Durum wheat and pasta at 30^{o}C

* Solvents A and B used for varietal identification of UK wheat were constantly purged with helium. This was found to reduce interrun variation.

2.6.3. Computation

Data aquisition was by means of a Nelson Analytical 900 series interface box, manipulation and storage was facilitated by the use of a Walters personal computer using Nelson Analytical software. Chromatograms of stored data were displayed on a video monitor using a Hercules graphics card. Peak areas were determined by a manual integration process on the monitor screen, by aligning the cursor at positions corresponding to the beginning and the end of an eluting peak. The area under this curve was calculated thus permitting exact comparisons to be made between the same time intervals in different chromatograms, ensuring a reproducible calculation of peak areas.

2.7. PROTEIN ESTIMATION ASSAY

The protocol undertaken was a modification of the method of Lowry *et al*. (1951), and used SDS at a final concentration of 0.8% (w/v) to aid in the solubilisation of otherwise insoluble proteins.

Stock solution A comprised of 4% (w/v) NaOH, 2% (w/v) Na_2CO_3, and 0.02% (w/v) sodium tartrate; stock solution B comprised of 0.5% (w/v) $CUSO_4.5H_20$; both solutions were prepared in distilled water. The sample protein was diluted to come within the bovine serum albumin range (50-1000 μg ml^{-1} then duplicate 100μl aliquots were mixed with 100μl aliquots of 10% (w/v) SDS. The stock solutions were mixed at a ratio of 12.25 A : 0.25 B and 1.0ml aliquots of this solution added to the protein samples in SDS. The samples were then vortexed and incubated for 20 min at room temperature, then 0.1ml of Folin & Ciocalteu's phenol reagent (freshly diluted 1:1 with distilled water) was added. The samples were vortexed and incubated a further 20 min at room temperature before measurement of absorbance at 750ηm. Standards of bovine serum albumin in the range 50-1000μg per ml were treated similarly.

2.8. PRODUCTION OF POLYCLONAL ANTIBODIES

2.8.1. Preparation of Antigen

Total gliadin extracts and semi-purified RP-HPLC separated gliadin fractions were prepared for the immunisation of rabbits as outlined in section 2.3.1.

2.8.2. Immunisation Protocol

The immunisation procedure outlined by Hudson and Hay (1980) was followed. Rabbits were injected subcutaneously into three separate sites. The initial immunisation was with a water and oil emulsion containing 500μg gliadins in 500μl of 10mM acetic acid mixed dropwise with 500μl of Freunds complete adjuvant (425μl of paraffin oil, 75μl of mannitol monolaureate, containing 500μg of mycobacterium tuberculosis M37 Ra, 17TCC 25177). This was followed by three further subcutaneous immunisations, at three week intervals with 250μg gliadins in 500μl of 10mM acetic acid mixed with 500μl of Freunds incomplete adjuvant (containing 425μl of paraffin oil and 75μl of mannitol monolaureate). Two control rabbits were simultaneously immunised with 5mM acetic acid in the appropriate Freunds adjuvant.

2.8.3. Test Bleeding, Monitoring and Immune Response

Two weeks after each successive immunisation, approximately 0.5ml of blood was taken from the marginal ear vein and dispensed into microcentrifuge tubes for clotting and serum isolation. Serum collected from respective rabbits was assayed for IgG directed to gliadins using a non-competitive ELISA. Serial thousand-fold dilutions of the serum were assayed along with control serum diluted similarly. Each dilution was assayed in triplicate.

2.8.4. Collection of Hyperimmune Anti-Serum

At the end of the immunisation protocol, when each rabbit had received 4 immunisations of each antigen solution, animals were anaesthetized and exsanguinated by cardiac puncture and severence of the neck arteries.

Rabbits were anaesthetized with a first intraperitoneal injection (2.0mg/Kg) of Hypnovel (5mg/ml Midazolam and 5mg/ml Midazolam base) followed by 0.3 ml/Kg Hypnorm (fentamyl citrate 3.5mg/ml and fluanisone 10mg/ml) injected into the marginal ear vein using a 2 ml syringe with a 0.5 mm bore needle. After 15-30 min, blood was removed by cardiac puncture until the heartbeat was no longer detectable and no more blood could be aspirated. The animal was killed by decapitation allowing further collection of blood from the neck arteries. The amount of blood obtained varied from 60 ml to 120 ml per rabbit. Blood was allowed to clot and serum was collected, aliquoted and stored at -20^{o}C prior to further processing.

2.9. ENZYME LINKED IMMUNOSORBANT ASSAY

2.9.1. Heterologous Non-Competitive Solid-Phase ELISA

All enzyme-linked immunosorbant assays were performed in 96 well polystyrene microtitre ELISA plates using a modification of the method of Engvall and Perlmann (1972) described in the following sections.

2.9.2. Solutions

The following solutions were made up in double distilled water unless otherwise stated:

Phosphate Buffered Saline (PBS) (0.14M NaCl)	8g sodium chloride 0.2g potassium chloride 1.15g anhydrous disodium hydrogen phosphate 0.2g potassium dihydrogen phosphate to 1L pH 7.4.
Poly-L-Lysine Solution	10µl Poly-L-Lysine (10mg ml^{-1} solution) in 10ml PBS
Coupling Buffer	50µl of glutaraldehyde in 10ml PBS (cold)
PBS-Tween	50µl Tween20 in 100ml PBS
Blocking Agent (1) (0.1M glycine)	2g "marvel" 0.75g glycine in 100ml PBS
Blocking Agent (2)	2g "marvel" in 100ml PBS
Anti-rabbit HRP Conjugate	1:1000 dilution of HRP anti-rabbit IgG in blocking agent 2
Anti-rabbit HRP substrate	20 ml 0.1M sodium acetate pH 6 150µl of TMB solution (10mg TMB in 1ml DMSO) 25µl 3% (w/v) hydrogen peroxide

2.9.3. Coating Wells With Gliadins

Aliquots (50μl) of poly-L-lysine solution (1mg 100 ml^{-1}) were dispensed into the wells of a clean ELISA plate, and incubated at room temperature. After 1h the wells were aspirated and washed once with PBS. The antigen solution (1μg gliadin in 50μl PBS per well) was added to the plate and allowed to react at room temperature. After 0.5h, 50μl of 0.05% (v/v) glutaraldehyde coupling buffer (mixed over ice) was added and the plate incubated at room temperature for a further 0.5h. The wells were again aspirated and washed twice with PBS. Wells were then filled with blocking solution (1) and incubated at 37^{o}C for 1h. Blocking solution 1 was replaced with blocking solution 2 and the plate was incubated at 37^{o}C for a further hour. When blocking was complete the wells were aspirated and washed with PBS-Tween 2-3 times followed by 2 washes of dH_2O.

2.9.4. ELISA Procedure

After blocking, the antigen coated plates were either used immediately or wells were filled with fresh blocking solution 2 and stored at -20^{o}C until required. The primary antibody solution (50μl), diluted appropriately in blocking solution 2 was added to each well and the plate incubated overnight at 4^{o}C. Wells were then aspirated and washed with PBS-Tween 2 or 3 times followed by two washes of distilled water. To each well, 50 μl of anti-rabbit HRP-IgG conjugate (diluted 1:1000 in blocking agent 2) was added followed by a 2h incubation at 37^{o}C. Wells were washed with PBS-Tween and distilled water as before. To each well 100μl of the anti-rabbit HRP substrate solution was added and the

reaction timed until the chromogen had produced a detectable blue colouration (15-20 min). The reaction was stopped by the addition of 50μl of 2.5M H_2SO_4 producing a yellow colour. Absorbance values were determined at 450 ηm using an ELISA reader. Control wells without antigen, without antibody and without antigen and antibody were included in each assay.

2.10. ISOLATION OF ANTI-GLIADIN IgG

2.10.1. Separation of IgG From Rabbit Anti-sera

The IgG fraction of immune and non-immune anti-sera obtained from rabbits was purified as described by Johnstone and Thorpe (1987). Ammonium sulphate (to a final concentration of 50% salt) was added over a 30 min period to the serum solution (25ml) mixing on an ice bath. The mixture was stirred on ice for a further 30 min followed by centrifugation at 3000 x g for 30 min at 4°C. The pellet was then collected, washed with 50% saturated ammonium sulphate (12ml) and recentrifuged. The washing procedure was repeated three times until each serum preparation was devoid of red cells. The pellet was then dissolved in 12 ml of 10mM potassium phosphate pH 8.0 and dialyzed at 4°C against 1000 ml of distilled water for each serum preparation. After 24 h each dialysis sac was placed in 1000ml of 20mM potassium phosphate pH 8.0 buffer and dialysed for a further 24 h at 4°C. At the end of this period the retentate was centrifuged at 3000 x g for 30 min to remove lipoprotein and the supernatant was stored at -20°C until required.

2.10.2. Preparation of Immunospecific Anti-γ-Gliadin IgG

2.10.2.1. Coupling of D. Valdur Gliadins to CnBr-activated Sepharose 4B

The procedure used was described by Johnstone and Thorpe (1987). Dry Cyanogen bromide activated sepharose 4B powder (2g) was pre-swollen in 1mM HCl for 15 min at room temperature, washed in 500ml of 1mM HCl and the washings removed by filtering on a sintered glass funnel. Durum gliadins (35mg) in 10mM acetic acid were added to 10ml of

coupling buffer (consisting of 100mM $NaHCO_3$ and 500mM NaCl at pH 9.0) and the pH was quickly checked. Immediately after the addition of protein, the solution was added to 7ml of pre-swollen cyanogen bromide activated sepharose 4B. After reacting overnight with end-over-end rotation at 4°C, the mixture was transferred to a scintered glass funnel and the liquid phase removed by applying a vacuum. This solution, was retained and assayed for protein to assess the coupling efficiency. The gel was washed thoroughly with 250mM $NaHCO_3$ at pH 9.0 and then blocked for 2h with gentle agitation using 1M ethanolamine (20ml) at pH 9.0, as the blocking agent. After blocking was complete the liquid phase was separated from the gel by applying a gentle vacuum as before and protein non-covalently absorbed to the gel was removed by washing with alternating buffers of high pH (500mM $NaHCO_3$, pH 9.0) and low pH (100mM Borate and 1M NaCl pH 4.1). Finally the gel was washed with distilled water and stored in PBS.

2.10.2.2. Immunoadsorbant Chromatography

Semipurified anti-sera (albumin removed) prepared from anti-sera collected from rabbits immunised with total and RP-HPLC fractionated gliadins were mixed with Durum gliadin-bound sepharose 4B. However the gel (5ml) was first mixed at room temperature with non-immune IgG isolate (0.5ml of isolate in 5ml of PBS). In this way sites subject to non-specific binding on both the insoluble support and the ligand were blocked, and thus made unavailable for further reaction when the immune fraction was added. After 2h this solution was filtered and the gel washed with 100ml of PBS. The binding capacity of the affinity column for anti-gliadin IgG

was estimated from data obtained by titrating varying levels of antigen with a constant antibody concentration determined by ELISA. This showed that optimum binding occured between 5ml of Durum gliadin-bound sepharose 4B and 12.5ml of a 1/100 dilution of semipurified anti-total gliadin immume solution or 12.5ml of a 1/10 dilution of semipurified anti-RP-HPLC fractionated gliadins. After mixing, the antibody solution and gel were allowed to react overnight at 4^{o}C with end-over-end rotation. After this the liquid fraction was separated from the gel and retained for further analysis and gel was mixed with 10ml of PBS.

Protein estimations (Lowry et al., 1951) were carried out at all stages of the separation to assess separation efficiency.

2.10.2.3. Chemical Elution of Bound Antibody

The Durum gliadin-bound sepharose 4B with bound anti-gliadin IgG was packed into a 10ml plastic syringe (the end of which was plugged with glass wool). The gel was allowed to settle. Then it was washed with 10 x 1ml washings of PBS and the washings retained. Bound antibodies were eluted with 10 x 1ml aliquots of 250mM Glycine-HCl (pH 2.5) and further antibody elution occured after adding 10 x 1ml aliquots of 250mM Glycine-HCl (pH 2.5) in 10% (v/v) 1,4 dioxane. On elution each of these fractions was immediately adjusted to pH 7.0 by addition of 2.5M Tris. Fractions having absorbancy (A280) readings greater than 0.05 were mixed together and retained for immunoblotting with gliadin proteins.

2.11. ELECTROBLOTTING

Electroblotting of gliadins was performed using a modified version of the method of Towbin et al. (1979). The electroblotting buffer used was 70mM acetic acid / 192mM at pH 3.2. Gliadins extracted from wheat and pasta samples in 25% (v/v) aqueous 2-Chloroethanol, (section 2.3.1.3.) were separated by acid polyacrylamide gel electrophoresis as described in section 2.4.1. After electrophoresis the edge of the gel was cut to mark the position of the sample lanes. The gel was then immersed in 100ml of electroblotting buffer to allow partial removal of urea and to facilitate easy handling of the gel for the next stage. Meanwhile 18 sheets of Whatman no.1 filter paper were cut to a size just slightly larger than that of the gel and soaked in the electroblotting buffer. Air bubbles were removed by gently rolling a clean glass rod over the sheets. Wearing a clean pair of gloves a sheet of nitrocellulose (pore size 0.45 μm) was cut to the size of the filter papers and also soaked in electroblotting buffer.

The Multiphor 11 semi-dry electroblotting apparatus was set up using the following procedure. On the lower graphite plate nine sheets of pre-soaked filter papers were placed, on top of which the pre-soaked sheet of nitrocellulose was applied. The equilibrated gel was carefully lifted onto the sheet of nitrocellulose and the remaining filter papers were placed on top of this. Air bubbles were excluded, by gently rolling the "sandwich" with a clean glass rod. The remaining equipment was assembled according to the manufacturers instructions. As gliadin

protein migrates towards the cathode at pH 3.2, the electrical connections were made in reverse to that used by Towbin et al. (1979). Electroblotting was carried out at room temperature with a current of 0.6mA / cm2 for 2h. After transfer the gel was removed and stained with Coomassie blue and the nitrocellulose sheet was stained with ponceau red to assess protein transfer.

2.12. IMMUNOBLOTTING OF DURUM AND COMMON WHEAT GLIADINS

Immunoblotting was carried out on electrophoretically separated, nitrocellulose bound gliadin. First the remaining binding sites on the nitrocellulose were blocked and the ponceau red stain removed by incubating the blot in buffer A (100mM Tris-HCl; 150mM sodium chloride; 0.05% (v/v) Tween 80; 2% w/v marvel, pH 9.0) for 2 h at room temperature with gentle shaking. The blot was then rinsed with buffer B (50mM Tris-HCl; 200mM sodium chloride; 0.1% (v/v) Tween 80, pH 7.4). Rabbit anti-gliadin IgG, (anti-sera, semi-purified IgG or affinity purified IgG) was diluted in buffer A and incubated with the blocked blot overnight at $4^{\circ}C$ with gentle agitation. After incubation the blot was washed for 30 min with three changes of buffer B and transferred to a new incubation tray. It was then incubated with anti-rabbit IgG-biotin conjugate diluted 1:10,000 in buffer B containing 2% (w/v) Marvel, with gentle shaking for 2h at room temperature. Next the blot was washed for 30 min with 3 changes of buffer B and transferred to a new tray and then incubated, for 30 min with constant shaking at room temperature with HRP-avidin conjugate diluted 1:5000 in buffer B. The blot was rinsed with distilled water washed with buffer B for 20 min and then equilibrated with PBS for 10 min. Finally it was incubated with 4-chloronaphthol reagent at room temperature until mauve coloured bands appeared (10-15 min). The reaction was stopped by rinsing the blot with distilled water.

Chloronaphthol reagent was made up just prior to use as follows: 20mg 4-chloro-1-naphthol was dissolved in

2.5ml DMSO; this solution was added dropwise to 47.5ml PBS with constant stirring; to this solution, 15μl of 30% (w/v) hydrogen peroxide was added prior to immediate incubation with the blot.

2.13. STATISTICAL ANALYSIS

Statistical analysis of data was undertaken using the Minitab statistical package (supplied by Minitab Inc., Pennsylvania, USA). The 95 percent confidence interval (C.I.) of sets of means was calculated and a two sample t-test was used to compare the difference between two means, when $p < 0.05$ the difference was considered to be statistically significant. The relationship between several variables was measured by calculating the corrolation coefficient between the variables. Data in the text are expressed as Mean +/-SEM.

3. RESULTS

In this project RP-HPLC was investigated for its use in the identification of intervarietal and interspecies differences in wheat gliadins. Based on some of these differences, chromatographic and immunochemical methods are presented which are capable of detecting unique gliadins that serve as suitable indicators of hexaploid wheat adulteration of Durum wheat and Durum pasta products.

In section 3.1 it was shown how gliadin RP-HPLC profiles can be used to differentiate between U.K. recommended hexaploid wheat varieties. Using quantitative computer analysis of these chromatograms a means for determining the proportions of known wheat cultivars in a wheat admixture was outlined, this led to the development of a technique to detect hybrid purity in wheat. Using similar computer analysis it was shown how common wheat adulteration of Durum wheat meal can be detected and quantified using RP-HPLC alone or RP-HPLC in combination with electrophoresis.

In section 3.2 the application of gliadin RP-HPLC and/or gliadin electrophoresis in the detection and quantification of common wheat adulteration of pasta products processed at normal and at elevated temperature was investigated. The efficacy of this technique was then compared to the current methods used to detect adulteration.

In section 3.3. the biochemical characteristics; Electrophoretic mobilities, 2-D PAGE patterns, isoelectric points and molecular weights of RP-HPLC fractionated

hexaploid wheat specific γ/β gliadins were compared with γ/β-gliadins extracted from total gliadin extracts not subjected to RP-HPLC separation. Furthermore the results are discussed in relation to the work of Huebner et al. (1967); Ewart (1977); Bietz et al. (1977); Popineau and Pineau (1985).

In section 3.4 it was shown that polyclonal antisera obtained from rabbits, immunised with hexaploid wheat gliadins, cross reacted with hexaploid wheat and Durum wheat gliadins. When this antisera was affinity purified using immobilised Durum gliadins a fraction of the anti-γ-gliadin IgG remains unbound which was shown to cross-react with the majority of hexaploid wheat varieties. No cross reactivity was observed with Durum wheat. The reaction of this antibody solution with electrophoretically separated and nitrocellulose bound gliadins extracted from pasta samples showed using this technique that it was possible to identify traces of common wheat adulteration in pasta samples.

3.1. ANALYSIS OF WHEAT GLIADINS BY RP-HPLC

3.1.1. WHEAT CULTIVAR IDENTIFICATION

Reversed-phase high-performance liquid chromatography of gliadin proteins using columns with large pore size packings (300Å) was shown by Bietz (1983), to be a suitable alternative to gliadin electrophoresis for the fractionation of cereal storage proteins. Like hydrophobic interaction chromatography (Popineau et al., 1980) RP-HPLC exploits differences in the surface hydrophobicity of proteins to acheive fractionation, whereas most electrophoretic and chromatographic separations are based on differences in charge or molecular size. Resolution of RP-HPLC however equals that of most commonly used biochemical techniques and greatly surpasses that of HIC. Furthermore RP-HPLC allows separation and detection of microgram quantities of proteins as required in single kernel analysis.

For the identification of wheat cultivars there is a considerable volume of literature pertaining to the use of either starch gel electrophoresis (Wrigley and Shepherd, 1974; Autran and Bourdet, 1975), or acid polyacrylamide gel electrophoresis (PAGE), (Bushuk and Zillman, 1978; Draper, 1987), of alcohol soluble seed storage proteins (Cooke, 1988). Wrigley and Shepherd, (1973), have shown that these proteins are under direct genomic control. These authors also showed that wheat of the same cultivar grown under widely differing environmental conditions and with grain protein contents ranging from 10-27 percent had almost indistinguishable gliadin electrophoretic patterns.

Since the initial work of Bietz (1983) RP-HPLC has

been proposed for varietal identification of wheat grown worldwide (see section 1.5). Optimal extraction and operating conditions have been determined and a variety of commercially available columns have been evaluated (Bietz et al., 1984a; 1984b; Bietz and Cobb, 1985; Kruger and Marchylo, 1985a; Marchylo et al., 1988). RP-HPLC exhibits excellent resolution and reproducibility (Bietz, 1983; 1985a; Marchylo and Kruger, 1984; 1985; Huebner and Bietz, 1988) but the most attractive features of the technique are its rapidity, ease of quantitation and automation (Scanlon et al., 1989a).

The purpose of the following work was to show that RP-HPLC can be used for varietel identification of UK wheat.

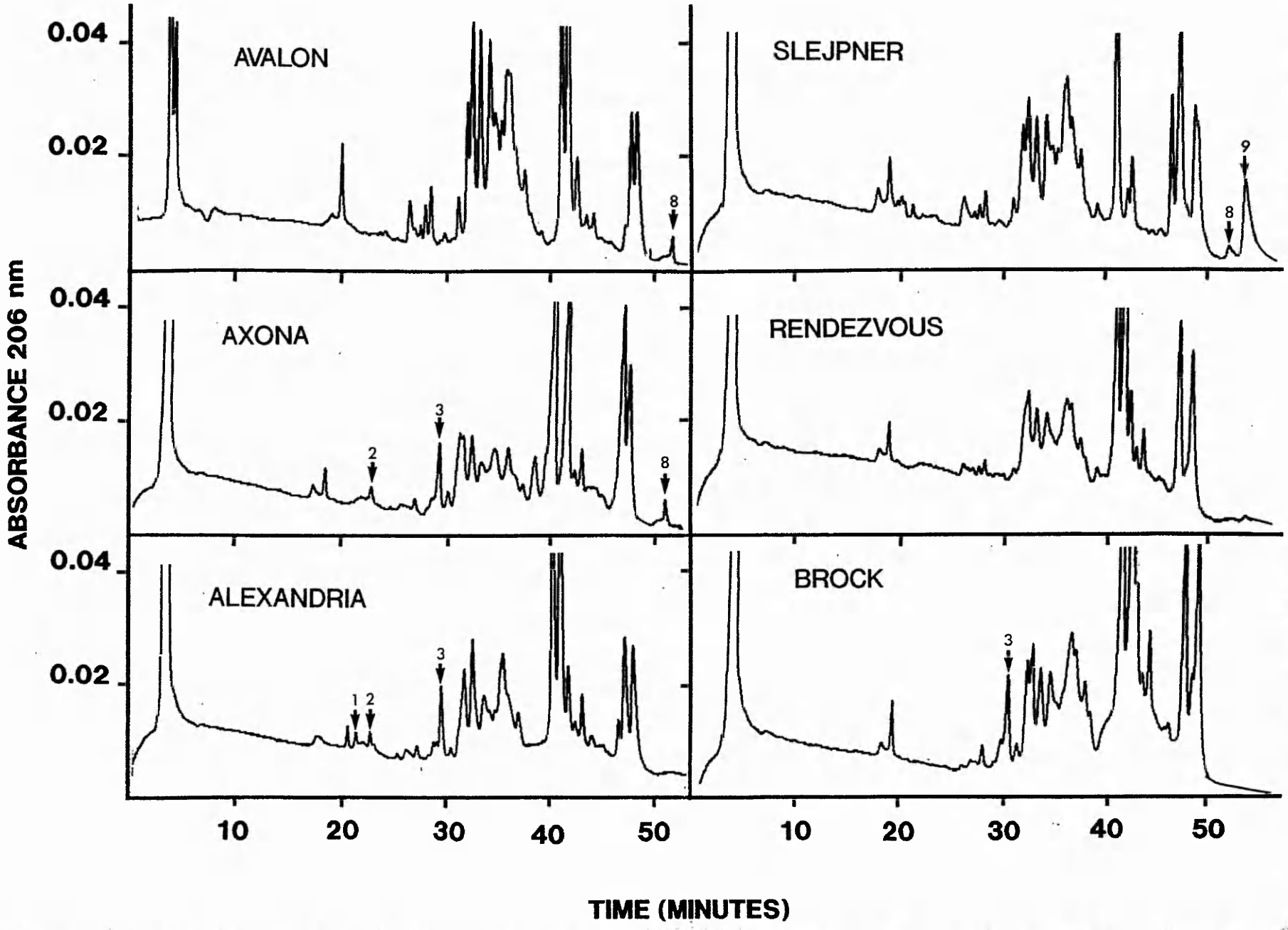
3.1.1.1. RP-HPLC Separation of Gliadins

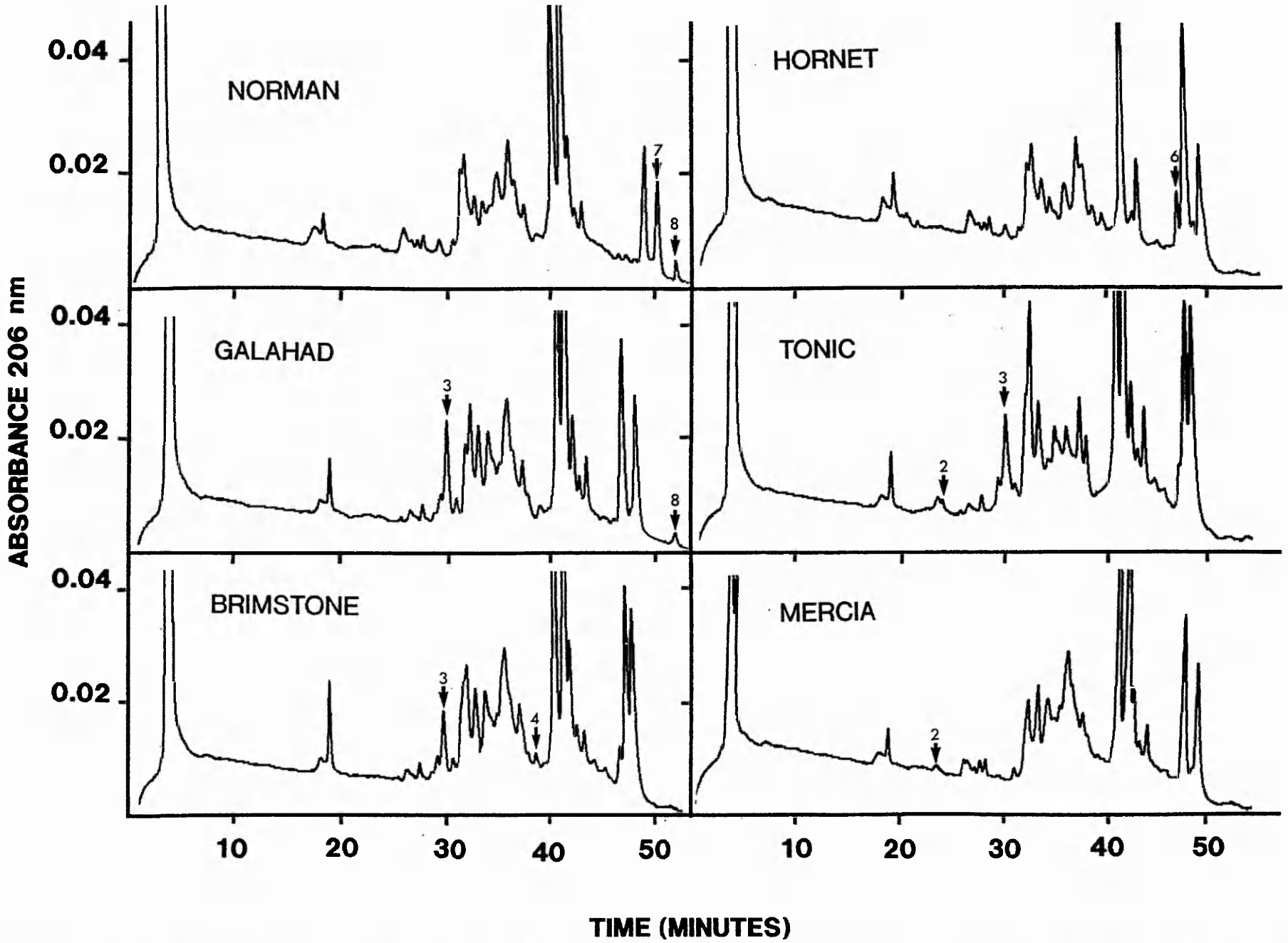
Figure 3.1. shows the gliadin RP-HPLC profiles for 18 UK hexaploid wheat varieties (Avalon, Axona, Alexandria, Slejpner, Rendezvous, Brock, Norman, Galahad, Brimstone, Hornet, Tonic, Mercia, Pastiche, Longbow, Wembley, Parade, Apollo and Riband) separated according to the procedure outlined in section 2.6. Gliadins of all varieties were fractionated into about 30 components, detected as peaks or poorly resolved shoulders. Proteins were eluted under very different acetonitrile concentrations (30-48%), indicating differences in surface hydrophobicity of gliadins. Repeated analysis of the same extract under the same conditions gave nearly identical chromatograms. Comparison of the different chromatograms revealed obvious significant and reproducible varietal differences among early-eluting, less hydrophobic components, as well as among late-eluting more hydrophobic components. Gliadin peaks, unique to different varieties are indicated by arrows and numbered 1-9 on Figure 3.1. The elution times of these peaks on different runs differed by no more than 25 sec as shown on Table 3.1.

The variation in the occurance of selected peaks in gliadin RP-HPLC chromatograms of these wheat varieties are summarised in Table 3.2. The presence or absence of a peak is indicated by either a plus sign or a minus sign respectively.

Figure 3.1 RP-HPLC Separation Profiles of Gliadin Extracts from Hexaploid Wheat Varieties.

Gliadin extracts (60μg protein) were loaded onto a Waters, Delta Pak, C18, 5μm particle size column (3.9x150mm), and then eluted according to the procedure outlined in section 2.6. The profiles illustrated are Avalon, Axona, Alexandria, Slejpner, Rendezvous, Brock, Norman, Galahad, Brimstone, Hornet, Tonic, Mercia, Pastiche, Longbow, Wembley, Parade, Apollo and Riband.


AVALON
SLEJPNER
AXONA
RENDEZVOUS
ALEXANDRIA
BROCK
0.04
0.02
ABSORBANCE 206 nm
1
2
3
8
9
10
20
30
40
50
TIME (MINUTES)


NORMAN
HORNET
GALAHAD
TONIC
BRIMSTONE
MERCIA
0.04
0.02
ABSORBANCE 206 nm
10
20
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40
50
TIME (MINUTES)

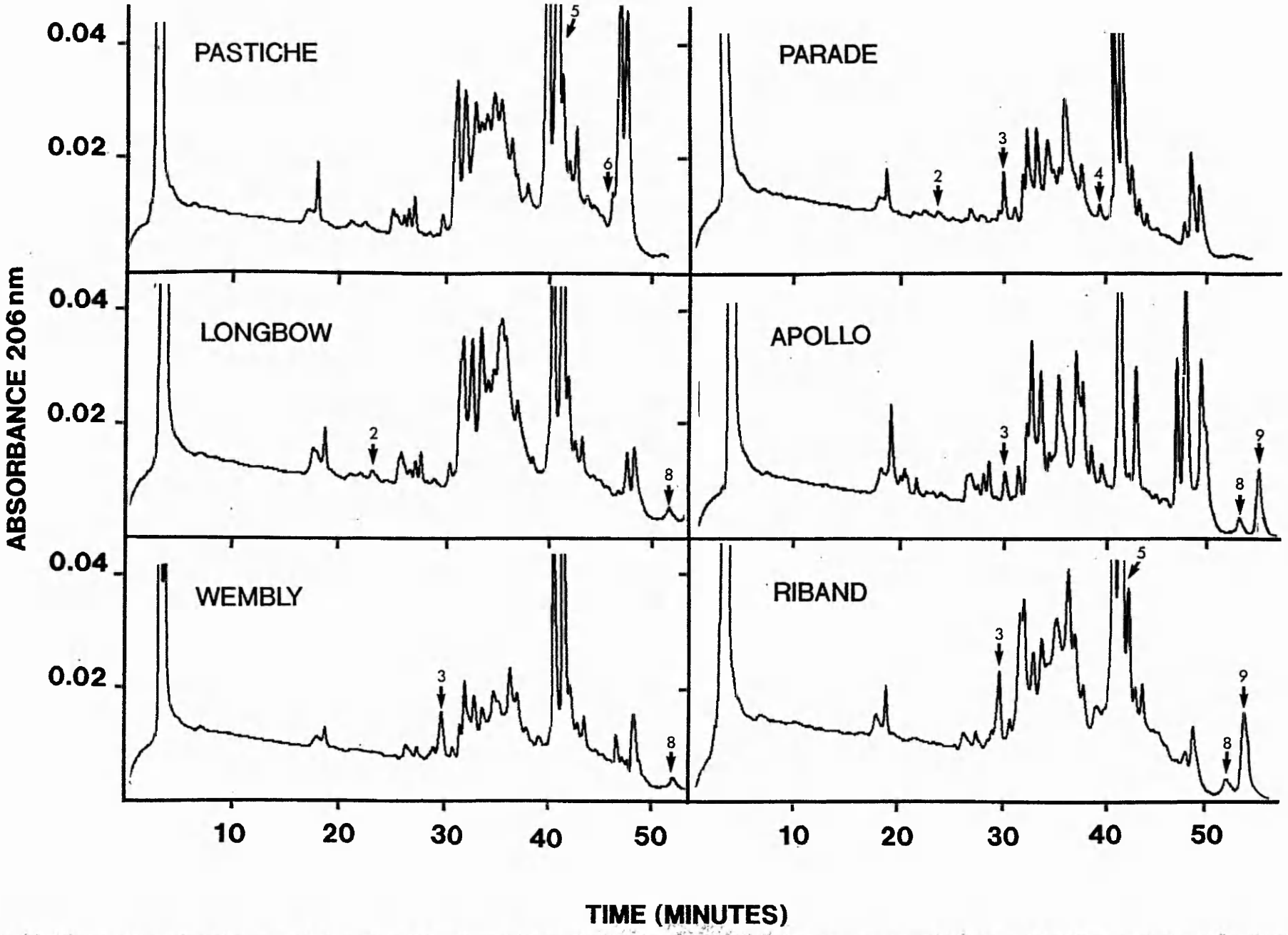

PASTICHE
PARADE
LONGBOW
APOLLO
WEMBLY
RIBAND
0.04
0.02
ABSORBANCE 206 nm
10
20
30
40
50
TIME (MINUTES)

Table 3.1. Mean+/-SE of Retention Times for Unique Gliadin Peaks used for Wheat Variety Identification (n=3).

peak	RT (min)	+/-SE (min)	RT Range (min)
1	21.30	0.16	21.14 - 21.46
2	22.40	0.29	22.11 - 22.69
3	29.70	0.27	29.43 - 29.97
4	38.21	0.12	38.09 - 38.33
5	41.36	0.18	41.18 - 41.54
6	46.34	0.48	45.86 - 46.82
7	49.72	0.40	49.32 - 49.12
8	52.78	0.39	52.39 - 52.17
9	55.04	0.70	54.34 - 55.74

Table 3.2. Variation in the Occurence of Selected peaks used for Wheat Variety Identification.

	Peaks								
	1	2	3	4	5	6	7	8	9
Avalon	-	-	-	+	+	+	-	+	-
Axona	-	+	+	+	+	-	-	+	-
Alexandria	+	+	+	-	+	+	-	-	-
Slejpner	+	+	-	+	-	+	+	+	+
Rendezvous	-	-	-	+	+	-	-	-	-
Brock	-	-	+	-	+	-	+	-	-
Norman	-	-	-	+	+	-	+	+	-
Galahad	-	-	+	+	+	-	-	+	-
Brimstone	-	-	+	+	+	+	-	-	-
Hornet	+	-	-	+	-	+	+	-	-
Tonic	-	+	+	-	+	+	-	-	-
Mercia	-	+	-	-	+	-	+	-	-
Pastiche	-	-	-	+	+	+	-	-	-
Longbow	-	+	-	+	+	-	-	+	-
Wembley	-	-	+	+	+	+	-	+	-
Parade	+	+	+	+	+	+	-	-	-
Apollo	+	-	+	+	-	+	+	+	+
Riband	-	-	+	+	+	-	-	+	+

Because most varieties possess a unique gliadin chromatogram the results demonstrate that RP-HPLC could be employed for wheat varietal identification. In addition an identification key is presented in Figure 3.2. which is based on qualitative characteristics (presence or absence of peaks); quantitative data, such as relative peak areas, could be used to further distinguish varieties. It can be seen that close similarities exist between the gliadin RP-HPLC profiles of some wheat varieties, for instance the varieties Tonic and Parade can only be identified by the presence of peak 4, a minor peak in the RP-HPLC profile of parade which is absent in Tonic. However similar varietal differentiation difficulties have been reported using the classification system resulting from the electrophoretic separation of gliadins (Autran and Bourdet, 1975).

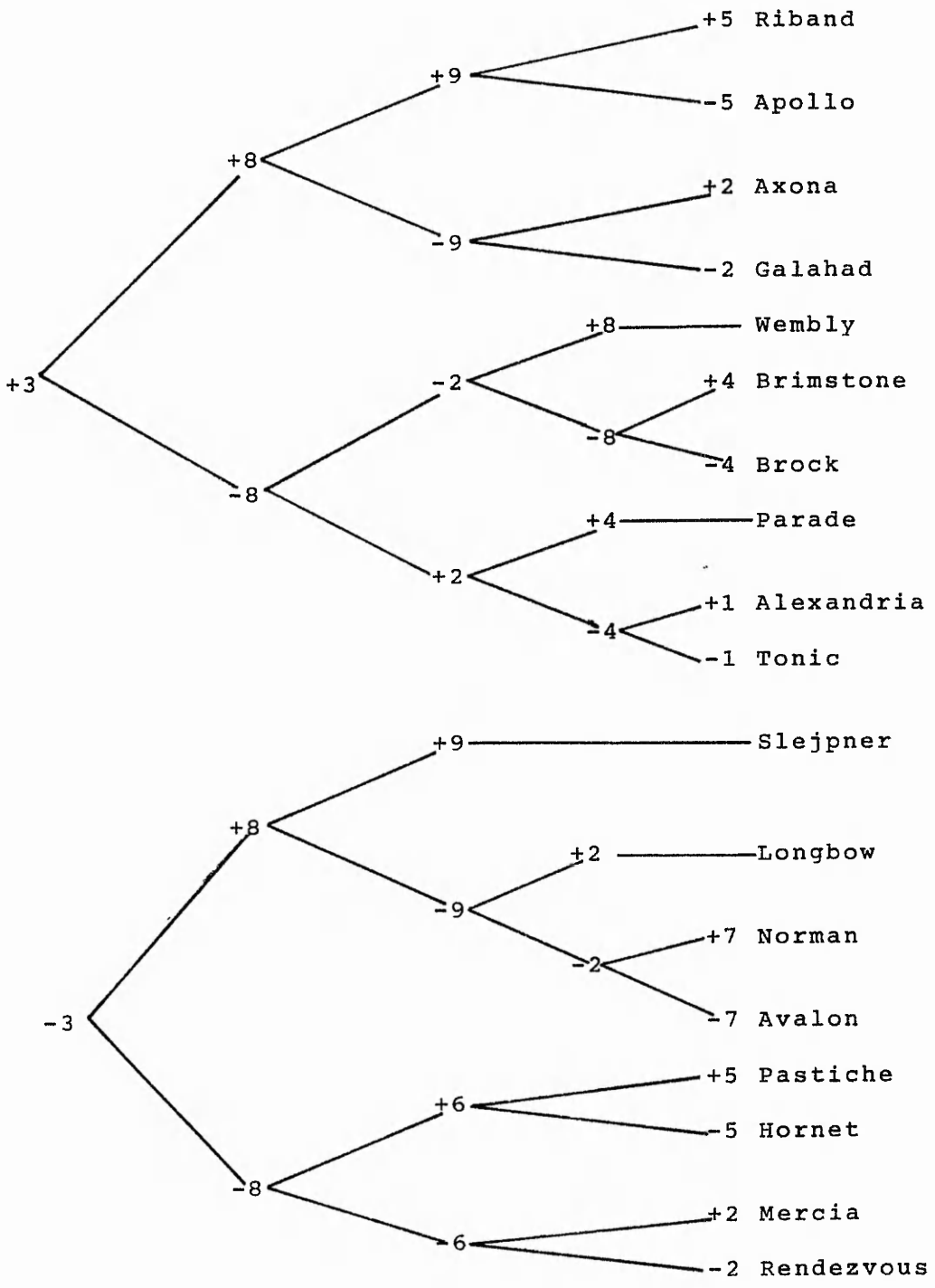
3.1.1.2. Discussion

This study demonstrates that polymorphism of gliadins as revealed by RP-HPLC can be used to identify UK wheat varieties. The method is rapid (analysis times of 1hr per sample), sensitive (determinations can be carried out on half kernels), reproducible and simple to operate. In addition, RP-HPLC can be automated and results can be accurately quantified.

The potential for routine manipulation of chromatogtaphic data exists but there have been few reports of computer-based methods for wheat protein analysis. Some workers have for example strived to use computers for predicting the proportion of wheat genotypes in simple known mixtures (Bietz and Cobb, 1985; Marchylo et al., 1988) for

Figure 3.2. Key to the Identification of 18 UK hexaploid wheat varieties

The classification is based on the presence or absence of chromatographic peaks separated by RP-HPLC on a Waters Delta Pak, C18, 5μm particle size column (3.9x150mm) and then eluted according to the procedure outlined in section 2.6.


+5 Riband
+9
-5 Apollo
+8
+2 Axona
-9
-2 Galahad
+8
Wembly
-2
+4 Brimstone
+3
-8
-4 Brock
+4
Parade
-8
+2
+1 Alexandria
-4
-1 Tonic
+9
Slejpner
+8
+2
Longbow
-9
+7 Norman
-2
-3
-7 Avalon
+5 Pastiche
+6
-5 Hornet
-8
+2 Mercia
-6
-2 Rendezvous

tracing pedigrees (Bietz and Huebner, 1987) and for estimating peak multiplicity (Bietz and Huebner, 1987). However, automated large scale comparison of chromatograms (computerised library searches) which is necessary for varietal identification is limited by the following problems: 1) the complexity of the RP-HPLC chromatograms of wheat from different genotypes (Marchylo et al., 1988; Wieser et al., 1987); 2) the variation of peak quantitation data from wheat grown in different locations (Huebner and Bietz, 1988); and 3) the changes in peak retention times with column use (Glajch et al., 1987; Marchylo et al., 1988; Scanlon et al., 1989a). Until these limitations are controlled computerised varietal identification based on RP-HPLC chromatograms is very difficult (Sapirstein et al., 1989).

The varietal identification key presented (Figure 3.2) is considered sufficient to discriminate between the 18 wheat cultivars on the UK wheat growers recommended list. For most laboratories this is the maximum number of varieties needed for routine identification analysis. Occasionally a greater number of varieties are needed. In this case a reliable computerised library search procedure which gives satisfactory discrimination has been proposed (Scanlon et al., 1989b). This involves the use of a computerised library of gliadin RP-HPLC fingerprint profiles comprising normalised peak retention times and relative peak heights (percent basis). These are used as primary and secondary discrimination parameters respectively. The computer scores peaks as being matched if both the retention times and relative peak heights are within prescribed difference

thresholds. The authors claim that the discriminatory power of the program is as good as computer-assisted varietal identification results based on gliadin PAGE and has the added advantage that no subjective input is required given the general quantitative capability of the chromatogram. However it is acknowledged that retention time drifts and column selectivity changes limit the versitility of the system.

At this point no relationship has been observed between the presence of any gliadin peak on the RP-HPLC profiles of these hexaploid wheat varieties and the analytical and/or technological characteristics in wheat. This concept has been explored further in the following sections.

3.1.2. ESTIMATION OF PURITY IN HYBRID WHEAT

In recent years a number of wheat breeders in Europe have resumed the investigation of the production of F1 hybrid wheat varieties. Based on the experience gained with other crops, an F1 hybrid variety normally has improved uniformity and resistance to particular diseases leading ultimately to an increased crop yield (Briggle, 1963). The production of F1 hybrid varieties requires the crossing of inbred parental lines, one of which (the female) can be rendered male sterile ie. produces no pollen. Interest is currently focussed on the use of chemical hybridising agents to induce male-sterility, (Bingham and Lupton, 1987) after which pollen from the chosen male line is used to fertilise the female line.

Using acid PAGE, the purity of an F1 hybrid wheat sample can be determined by identifying the gliadin pattern of each individual seed in a sample (Cooke, 1988). The simple Mendelian inheritance and co-dominant expression of gliadins (Lupton, 1987), means that F1 hybrid seed contain all of the gliadin proteins found in either the male or female parent. Seeds which do not contain all of these gliadins cannot be F1 hybrids. Hybrid purity can then be expressed as the percentage of seeds in a sample which are true F1 hybrids. Normally this means that at least 100 individual seeds have to be analysed to generate results within acceptable confidence limits (Draper, 1987).

Over the past six years the resolving power of RP-HPLC for the separation of cereal proteins has improved. It has been proposed (Bietz, 1985; Paulis *et al.*, 1986), as a

means of identifying maize inbreeds and maize hybrids and as shown in section 3.1.1 as a means of identifying wheat cultivars (Bietz et al., 1984b; Bietz and Cobb, 1985; Huebner and Bietz, 1986). More recently the quantitative aspects of the raw chromatographic data has been explored: Marchylo et al. (1988), have used a computer-based RP-HPLC method to predict the proportion of different wheat genotypes in an unknown mixture; Bietz and Huebner (1987), proposed a quantitative RP-HPLC method to predict wheat quality; more recently Scanlon et al. (1989b), developed a totally automated computerized method for wheat varietal identification.

The object of the following work was to demonstrate how RP-HPLC can be used to identify and characterise F1 hybrid wheat. Furthermore by comparing the technique to acid PAGE, the potential of RP-HPLC as a method which could be used for the rapid analysis of F1 hybrid purity was assessed.

3.1.2.1. Production of F1 Hybrids

The production of F1 hybrid samples, from the cross S♂ x S♀, was carried out under the supervision of the NIAB, as outlined diagramatically in Figure 3.3. The F1 hybrids used in this study were harvested from strips A and D at intervals marked 1-8, and designated SA1-SA8 and SD1-SD8.

▨ corresponds to blocks of female plants

□ corresponds to blocks of male plants

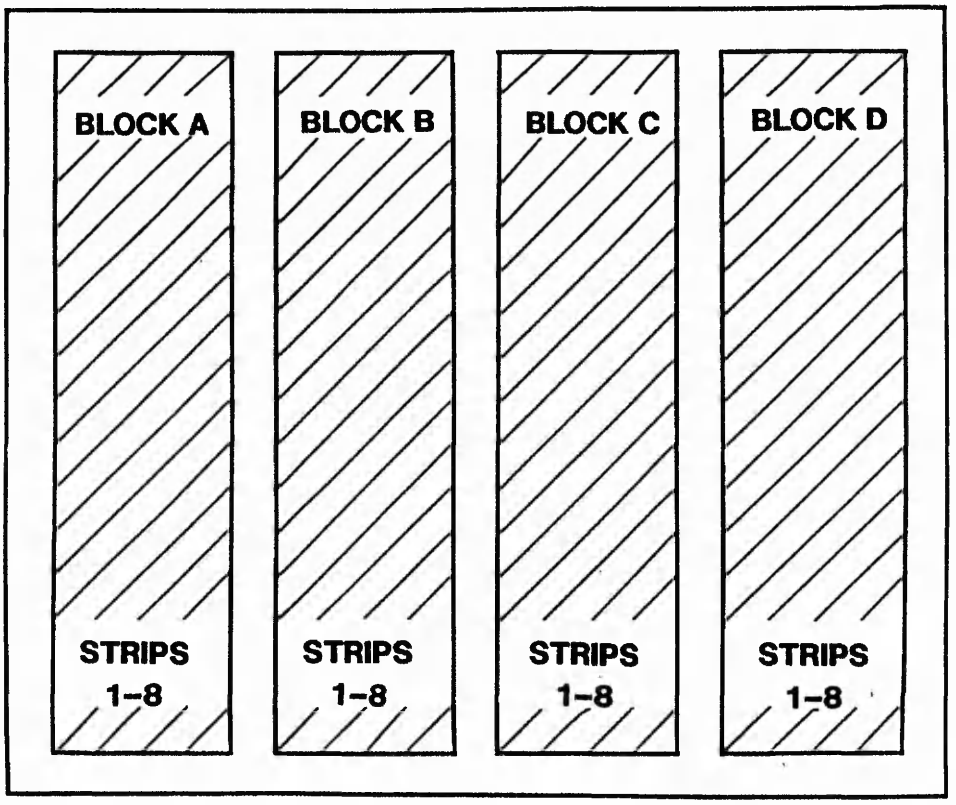


Figure 3.3 Diagramatic Representation of the Field Trial Set-up used for Growing F1 Hybrid Wheat

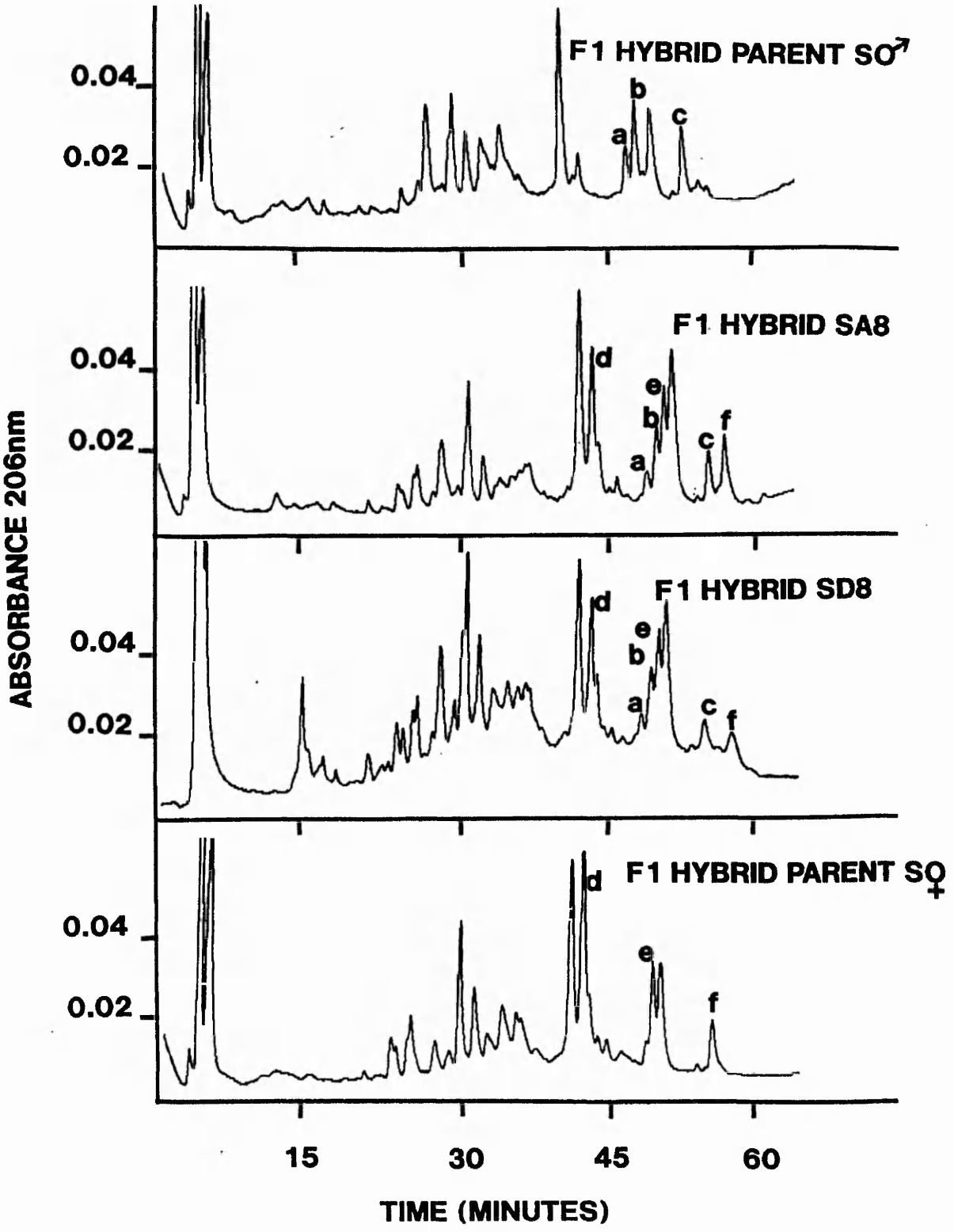
3.1.2.2. Estimation of F1 Hybrid Purity Using Gliadin RP-HPLC Profiles

The RP-HPLC gliadin elution profiles for the hybrid parents S♂ and S♀ and F1 hybrids SA8 and SD8 are shown in Figure 3.4. Repeated analysis of the gliadin extracts, under similar conditions (outlined in section 2.6.) gave nearly identical elution patterns which were characteristic for each parent. Elution times for the same peaks never differed by more than 25 sec between runs. In the elution profiles, characteristic peaks eluting between 40 and 60 min were recognisable which contain gliadins unique to each parent. For example, in Figure 3.4., the peaks eluting at 47.40 min (a), 48.20 min (b) and 53.00 min (c) represented gliadins unique to the male parent while the peaks eluting at 42.30 min (d), 47.00 min (e) and 55.00 min (f) represented gliadins unique to the female parent. All other peaks were common to both profiles. In some cases early eluting peaks (eluting at 15 min, hybrid SD8) were found in the chromatograms. These artefactual peaks were generally the result of incomplete column washing between separations. The RP-HPLC gliadin profiles obtained for single seeds of the F1 cross S♂ x S♀ taken from strips SA8 and SD8 were composed of peaks which were also present in the profiles of both parents. Furthermore the unique gliadin peaks characteristic of the male parent (eluting at 47.40, 48.20 and 53.00 min) and the female parent (eluting at 42.30, 47.00 and 55.00 min) were all present.

As expected from the known inheritance of gliadins and from electrophoresis, the gliadin RP-HPLC profile of an

Figure 3.4 Gliadin RP-HPLC Profiles of the F1 Hybrid Parents S♂ and S♀ and F1 Hybrids SA8 and SD8.

Gliadin extracts (100 μg of protein) were loaded onto a Waters, delta pak, C18, 5μm particle size column, and eluted using the procedure outlined in section 2.6. The profiles illustrated are (a) hybrid parent S♂ (b) F1 hybrid SA8 (c) F1 hybrid SD8 (d) hybrid parent S♀.


F1 HYBRID PARENT SO♂
F1 HYBRID SA8
F1 HYBRID SD8
F1 HYBRID PARENT SO♀
0.04
0.02
a
b
c
d
e
f
ABSORBANCE 206nm
15
30
45
60
TIME (MINUTES)

F1 hybrid is a combination of the gliadin profiles of both parents as separated using a 55 min aqueous acetonitrile gradient. It should therefore be possible to analyse individual seeds from a sample of F1 hybrid wheat and determine the percentage purity of the sample by identifying and rejecting seeds whose gliadin RP-HPLC profile clearly is not a combination of the gliadin RP-HPLC profiles of each parent and hence not an F1 hybrid. In this way the percentage of true F1 hybrid seeds may be determined. Shorter analysis times (15 min per seed) are possible, giving adequate resolution (Bietz and Cobb, 1985), and unlike electrophoresis the technique can be fully automated. However such an approach which still requires the analysis of approximately 100 individual seeds has little advantage, with regard to speed, over the use of acid PAGE.

3.1.2.3. Development of a Rapid RP-HPLC Method to Estimate Hybrid Purity

If a more rapid determination is essential, then a quantitative technique capable of determining the hybrid purity of a batch sample of F1 seeds is required. It is now well documented (Mecham et al., 1978) that the F1 hybrid inherits two doses of genetic material from the female parent and one dose from the male parent. Hence in the RP-HPLC elution profile of gliadins extracted from an F1 hybrid, the integrated area of the unique gliadin peaks (representing gliadins inherited from the female and male parents) should be 2/3 and 1/3 the area of these peaks found in the gliadin RP-HPLC profile of the female and male parents respectively. Any deviation from this allocation, when calculated for a

batch sample of seeds, would indicate an impure F1 hybrid wheat sample.

However when unique peaks on the gliadin RP-HPLC profiles obtained from single seed extracts of both parents (n=5) were compared quantitatively with similar peaks obtained from the elution profiles of extracts of single F1 hybrid seeds (n=5) this allocation of gene dosage often proved difficult to substantiate using peak areas alone (from a number of different chromatograms). Such a method required the injection of equimolar concentrations of protein from the different samples and careful monitoring of the sample volume, if accurate quantitative measurements of peak areas were to be made (Kruger and Marchylo, 1985a; Huebner and Bietz, 1988; Marchylo and Kruger, 1988). Considering the many variables that may be introduced into the extraction procedure and during sample injection, such stringency would be difficult to maintain in the quality control laboratory, Thus an alternative method of assessing hybrid purity was sought.

The main impurity present in an F1 hybrid seed sample will occur either as a result of cross pollination from other wheat (in addition to that of the male parent) or self pollination of the female (because of incomplete sterilisation of the anthers). In either case the genetic dosage of the female parent will remain constant regardless of the hybrid purity. However the genetic dosage contributed by the male parent will vary according to the purity of the sample. Hence any changes in the unique gliadins contributed by the male parent should be indicative of the purity of that

F1 hybrid wheat sample.

To test this experimentally, gliadins were extracted from four wheat mixtures made up to represent hybrids of varying purity, ie. 100%, 90%, 75% and 50% hybrid purity (Figure 3.5.). In each admixture the percentage of seed (66.70%) from the artificial female parent was kept constant. The remaining percentage of seed (33.30%) included seed from an artificial male parent, with varying levels of contamination (0.00-16.66%) represented by an admixture containing equal amounts of Avalon, Hobbit, Moulin and Maris Huntsman. The gliadin RP-HPLC profile of an authentic F1 hybrid wheat was deduced from profiles obtained from six randomly chosen F1 hybrid seeds, when the elution profiles were found to be identical (Figure 3.6.). This profile was virtually identical to that obtained with the artificially prepared 100% hybrid (Figure 3.5.i) and was thus used as the 100% pure hybrid. By reference to this, seed admixtures and hybrid samples of unknown purity were compared.

The gliadin profiles of extracts of the artificial hybrid samples, shown in Figure 3.5 indicated that the size of the peaks unique to the male parent (peaks a, b and c) decreased proportionally as the percentage hybrid purity decreased. However the size of peaks unique to the female parent (peaks d, e and f) appeared unchanged. To obtain a quantitative result, peak area ratios were calculated for each of these hybrid admixtures. The use of such ratios avoids errors associated with gliadin extraction and injection. The ratio of peaks most suitable for determining F1 hybrid purity involved calculating the ratio between one

Figure 3.5 Gliadin RP-HPLC Profiles of Mixtures of F1 Hybrid Parents S♂ and S♀ Corresponding to Hybrid Purity between 100-50 Percent.

Chromatographic conditions used are outlined in section 2.6. using a column loading of 100 μg of protein. The profiles illustrated are for extracts of (i) an admixture corresponding to 100% hybrid purity (ii) an admixture corresponding to 90 percent hybrid purity (iii) an admixture corresponding to 75 percent hybrid purity (iv) an admixture corresponding to 50 percent hybrid purity.

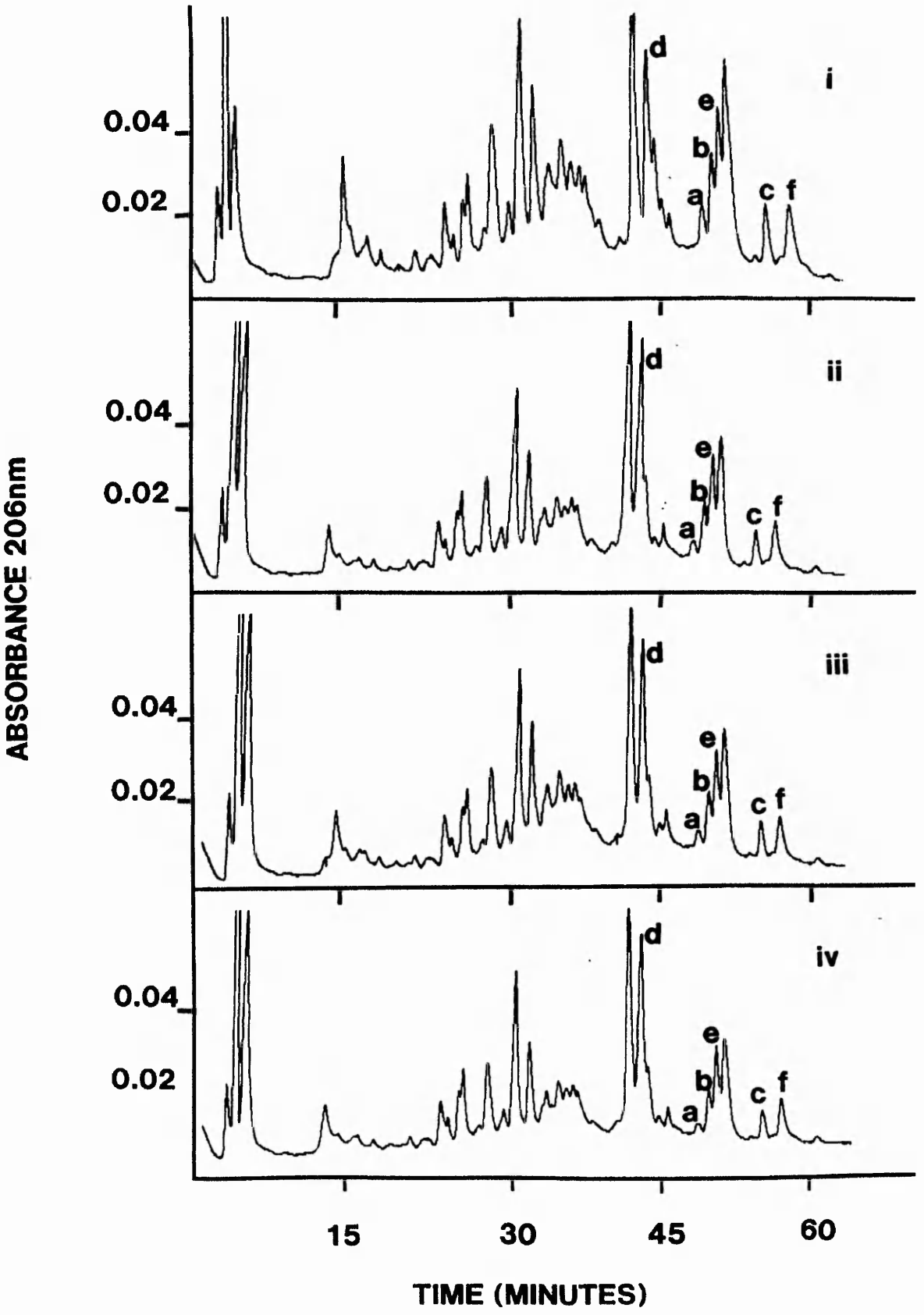
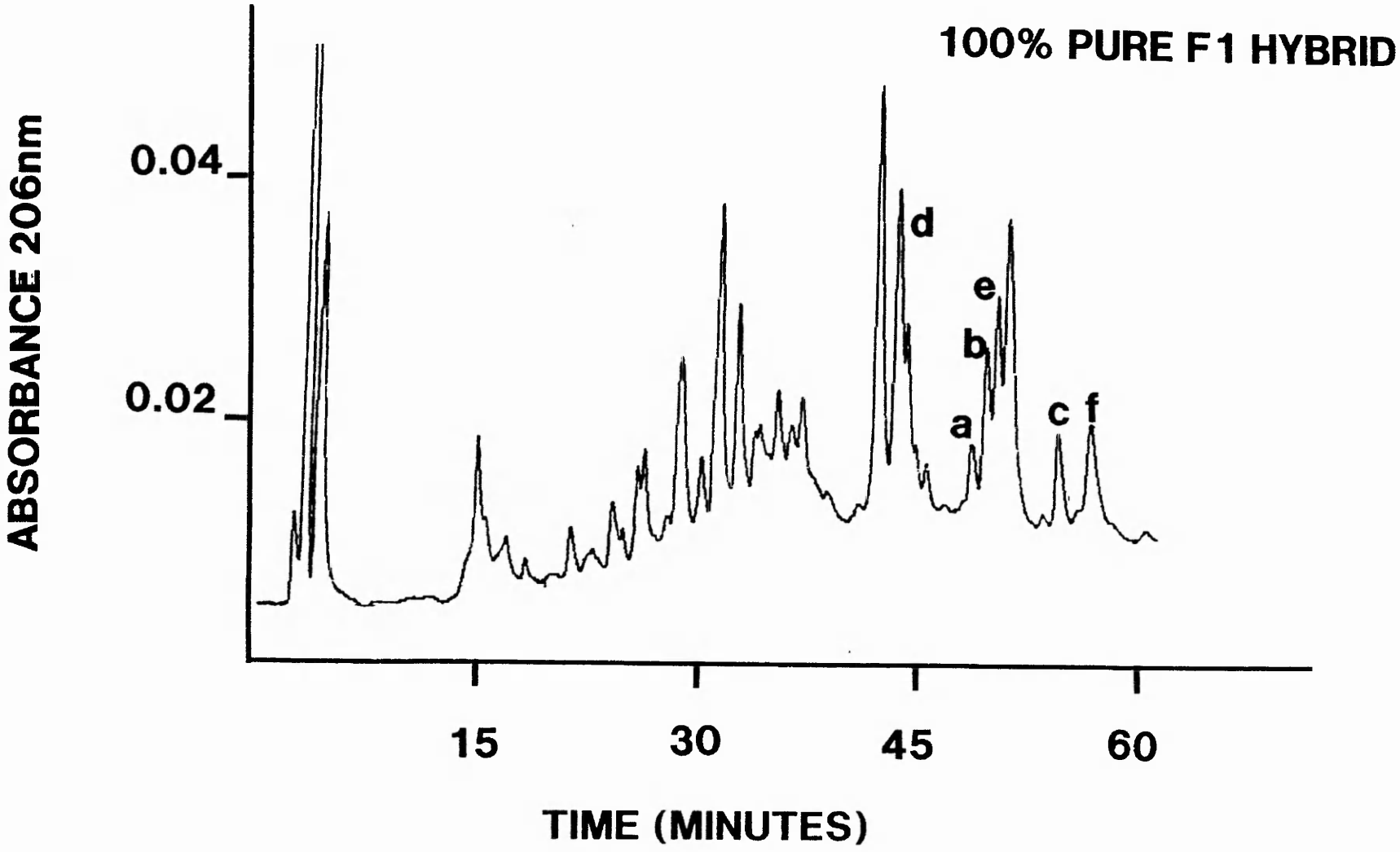

ABSORBANCE 206nm
0.04
0.02
i
ii
iii
iv
a
b
c
d
e
f
15
30
45
60
TIME (MINUTES)

Figure 3.6 Gliadin RP-HPLC Profiles of a True F1 Hybrid

Chromatographic conditions are outlined in section 2.6 using a column loading of 100 μg of protein. The Figure illustrates a single seed of the F1 hybrid SA1 (found to be a true hybrid).


100% PURE F1 HYBRID
ABSORBANCE 206nm
0.04
0.02
d
e
b
a
c
f
15
30
45
60
TIME (MINUTES)

peak unique to the male parent with a peak unique to the female parent. Peaks b and e were chosen for this study since they had the lowest peak area standard error calculated from nine different elution profiles. Ratio (peaks e/b) values were calculated, corresponding to hybrid purity between 100% and 50% (Table 3.3) and used to construct standard curves relating area ratio to hybrid purity. The ratio value for a pure F1 hybrid was taken from the analysis of single F1 hybrid seeds and not the 100% artificial admixture, although the values obtained for both were not significantly different. Separate standard curves (Figure 3.7) were prepared in conjunction with the analysis of both sets of hybrids, ie. SA1-SA8 and SD1-SD8. This was thought necessary to overcome any chromatographic variation resulting from column deterioration and solvent preparation since hybrid samples were analysed at different times corresponding to a seven day interval between analyses. However reference to Table 3.3 indicates little significant difference between the two sets of data.

3.1.2.4. Estimation of Hybrid Purity by RP-HPLC of Gliadins Extracted from Multi-seed Samples

To test this method as a means of determining hybrid purity, gliadins extracted from batch samples (100 seeds) of each of the F1 hybrids, SA1-SA8 and SD1-SD8, were separated by RP-HPLC. The gliadin separation profiles for two of these are shown in Figure 3.4. The peak area ratios (peaks e/b, shown on Table 3.3) of these were calculated and the percentage purity of each sample was extrapolated from the appropriate standard curve (Figure 3.7). For comparative

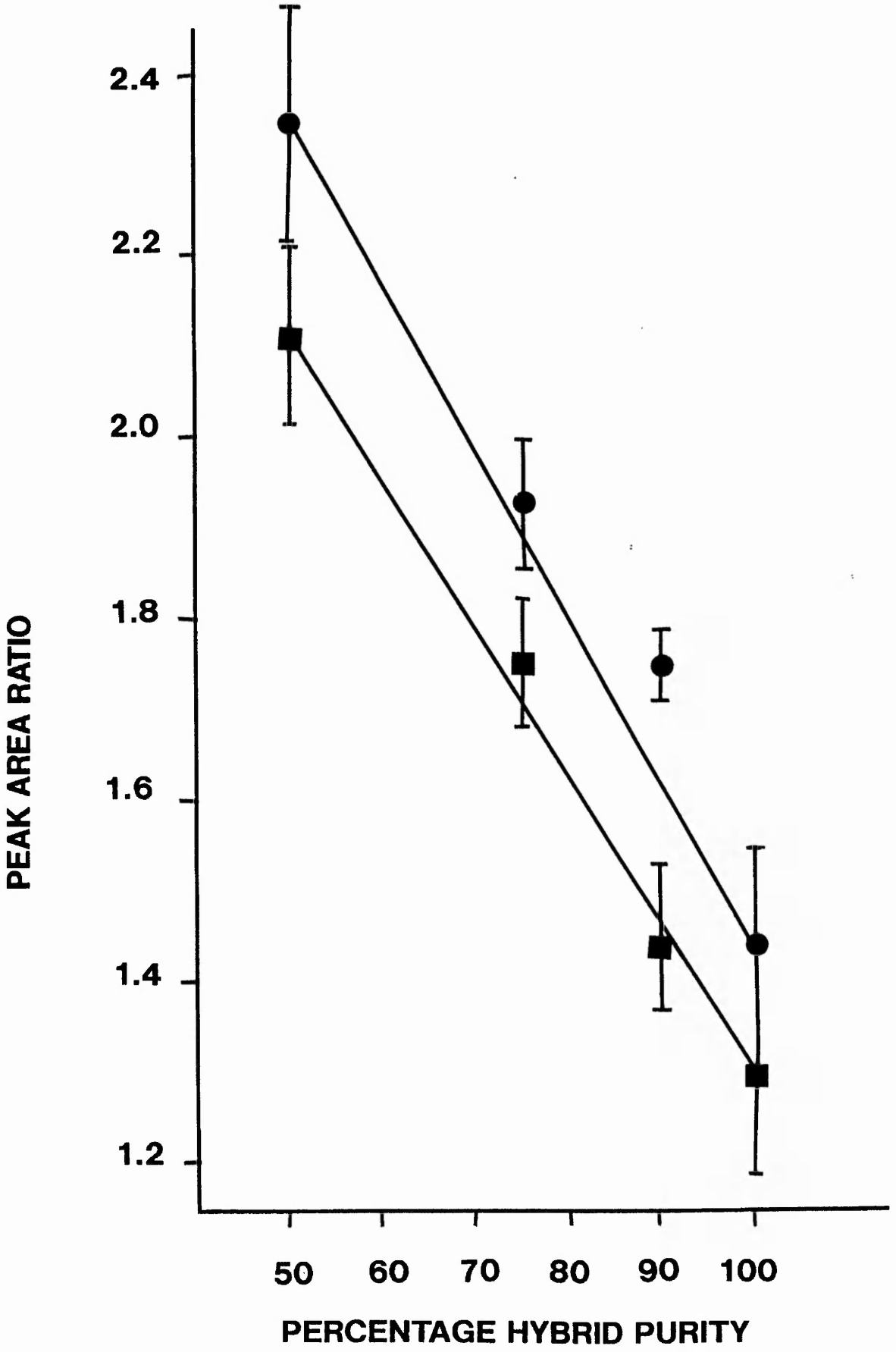
Table 3.3 Relationship Between Peak Area Ratios (peaks e/b) and Percentage Hybrid Purity for Gliadin Extracts of a True F1 Hybrid and Standard Wheat Mixtures of Known Purity, Separated by RP-HPLC.

Hybrid purity (percentage)	---SA Hybrid--- Peak Area Ratio (mean +/- SEM)	---SD Hybrid--- Peak Area Ratio (mean +/- SEM)	p Values
100 (n=5)	1.45 +/- 0.10	1.30 +/- 0.10	$p < 0.05$
90 (n=3)	1.75 +/- 0.04	1.45 +/- 0.08	$p < 0.10$
75 (n=3)	1.93 +/- 0.06	1.75 +/- 0.07	$p < 0.05$
50 (n=3)	2.36 +/- 0.14	2.12 +/- 0.10	$p < 0.05$

(ratio differences with a p-value < 0.05 are considered statistically significant).

Figure 3.7 Standard Curves Relating Peak Area Ratio and Percentage Hybrid Purity of Wheat Mixtures

Mixtures were prepared by varying the concentration of contaminating wheat mixture between 0.00-16.66 percent of the final mixture. Curve 1 (●) was obtained from samples prepared and analysed in conjunction with the SA F1 hybrids and curve 2 (■) was obtained from samples prepared and analysed in conjunction with the SD F1 hybrids. The 100 percent hybrid purity ratio value was taken from RP-HPLC gliadin profiles (n=5) of true F1 hybrid seeds.


2.4
2.2
2.0
1.8
1.6
1.4
1.2
PEAK AREA RATIO
50
60
70
80
90
100
PERCENTAGE HYBRID PURITY

purposes the percentage hybrid purity of each sample previously determined by acid PAGE (at NIAB) is shown in Table 3.4. Only in one F1 hybrid wheat (SA8) was the percentage hybrid purity as calculated from RP-HPLC data identical to that obtained using electrophoresis. In nine samples (SA1, SA2, SA5, SA6, SA7, SD2, SD3, SD4 and SD8), the F1 hybrid purity as calculated by both methods differed by 6% or less, in a further 5 samples (SA3, SD1, SD5, SD6 and SD7) a difference of 11% or less was observed (Table 3.4). In one sample (SA4) the difference observed between methods was 14%. Thus it is evident that this approach may represent an alternative way of estimating hybrid purity which is considerably more rapid than single seed analysis by PAGE or RP-HPLC.

3.1.2.5. Discussion

RP-HPLC separation of wheat gliadins has been used successfully for wheat variety identification (Bietz et al., 1984b; Bietz and Cobb, 1985; Scanlon et al., 1989b). The present data indicate that this separation technique can be used for F1 hybrid purity determination, by characterising unique peaks in F1 hybrid wheats, which represent gliadins inherited from either the male or female parent. Using this protocol two alternative approaches were possible. The first approach involved a RP-HPLC separation (using a 10 min acetonitrile gradient), of wheat gliadins extracted from 100 individual seeds (Bietz and Cobb, 1985). After comparison of the individual profiles with those of the parent wheats, the percentage of F1 hybrid seeds in the admixture was calculated. Using this technique 100 seeds could be analysed

Table 3.4 Purity Analysis of F1 Hybrids SA 1-8 and SD 1-8.

Hybrid (n=3)	---RP-HPLC--- Hybrid Purity (%)	---Acid PAGE--- Hybrid Purity (%)	Difference (%)
SA1	90	86	4.5
SA2	82	80	2.5
SA3	88	80	9.0
SA4	88	76	14.0
SA5	81	86	6.0
SA6	89	86	3.0
SA7	89	88	1.0
SA8	91	91	-
SD1	92	82	11.0
SD2	83	82	1.0
SD3	85	84	1.0
SD4	83	82	1.0
SD5	85	77	9.5
SD6	89	82	8.0
SD7	83	90	8.0
SD8	89	87	2.0

Hybrid Purity was determined using (a), data collected on the RP-HPLC separation of gliadins, extracted from a representative mixture of seeds (100 seeds) and (b) data collected from the acid PAGE separation of gliadins extracted from single seeds in a representative sample of seeds (100 seeds).

over a 25h period. This was broadly comparable to the time taken for analysing the same number of seeds using acid PAGE (Draper, 1987) if gel staining and evaluation times were taken into consideration.

The second technique involved the RP-HPLC separation (in triplicate) of a random sample (containing at least 100 seeds) of F1 hybrid seeds. Unique peaks representative of gliadins inherited from either the male or female parent were identified on the RP-HPLC profile of the F1 hybrid wheat. The peak area ratio of these peaks was calculated and then compared to a standard curve equating peak area ratios and percentage F1 hybrid purity. From this curve the hybrid purity of the unknown sample was determined. Using this technique a hybrid sample could be analysed in less than 4h and the system could be fully automated. When this technique was applied to F1 hybrid samples of unknown purity (SA and SD hybrids) the results were comparable to those obtained by electrophoretic analysis of individual seeds.

The main source of error associated with this technique was in measuring peak areas. This error was less in screen monitored computerized integrators as peak areas could be determined reproducibly on the video monitor by aligning the cursor at positions corresponding to the beginning and the end of each peak. The area under this curve could be calculated, thus permitting exact comparisons to be made between the same time intervals in different chromatograms. It has been suggested by Marchylo and Kruger (1988), that error increases as the sample volume increases,

as progressively more gliadin proteins do not bind to the column. Common to all RP-HPLC separations the assay exhibited week to week variation which made regular measurement of standards necessary.

RP-HPLC profiles of wheat storage proteins reflect the genetic exchanges known to occur during crossing. In addition, these genetic exchanges give rise to characteristic gliadins in the F1 hybrid which may be successfully used as a rapid means of measuring hybrid purity in a sample. As potential applications of RP-HPLC for the separation of wheat proteins increases it may be possible to use some profiles for advanced and accurate prediction of functional properties of wheat.

3.1.3. DETECTION AND QUANTIFICATION OF COMMON WHEAT ADULTERATION OF DURUM WHEAT

A large proportion of European pasta products on sale are prepared from Durum wheat (Triticum Durum) only. Italian law lays down rules for the manufacture and marketing of pasta products and prohibits the sale of products made from hexaploid wheat (Triticum Aestivum) or a mixture of hexaploid and Durum wheat. Extension of this prohibition to imported products has been ruled incompatible with articles 30 and 36 by the European court (C215/09, C215/10 1988). Owing to price differentials between types of wheat it is most likely that an increasing number of pasta products prepared from mixtures of Durum and hexaploid wheat will be traded.

Currently Durum wheat flour (and pasta derived from it) is tested for adulteration with common wheat using either one or a combination of electrophoretic or tlc separation procedures (section 1.1.6.3.). These procedures distinguish the two species by identification of components normally only found in hexaploid wheat varieties and whose presence in Durum wheat is considered indicative of adulteration. The methods have been found to be accurate and complimentary with remarkable reproducibility. However the procedures are tedious and not easily automated. In addition interpretation is highly subjective and therefore results are mainly used for a qualitative assessment with any quantitative measurements being at best an estimate.

In section 3.1.1 the use of gliadin RP-HPLC profiles for wheat varietal identification was outlined and

in section 3.1.2 it was shown how this essentially quantitative technique also reflects wheat genetic differences, which previously had only been shown using electrophoresis. RP-HPLC and electrophoresis separate proteins by different criteria but Bietz (1983) and Lookhart et al. (1986) have shown that the methods are highly complimentary. In the following study RP-HPLC and RP-HPLC combined with electrophoresis was used to identify gliadins (25% v/v 2-chloroethanol extractable) from different varieties of hexaploid wheat and Durum wheat. These differences were then used to develop methods which can detect the adulteration of Durum wheat with levels of hexaploid wheat as low as 1 percent.

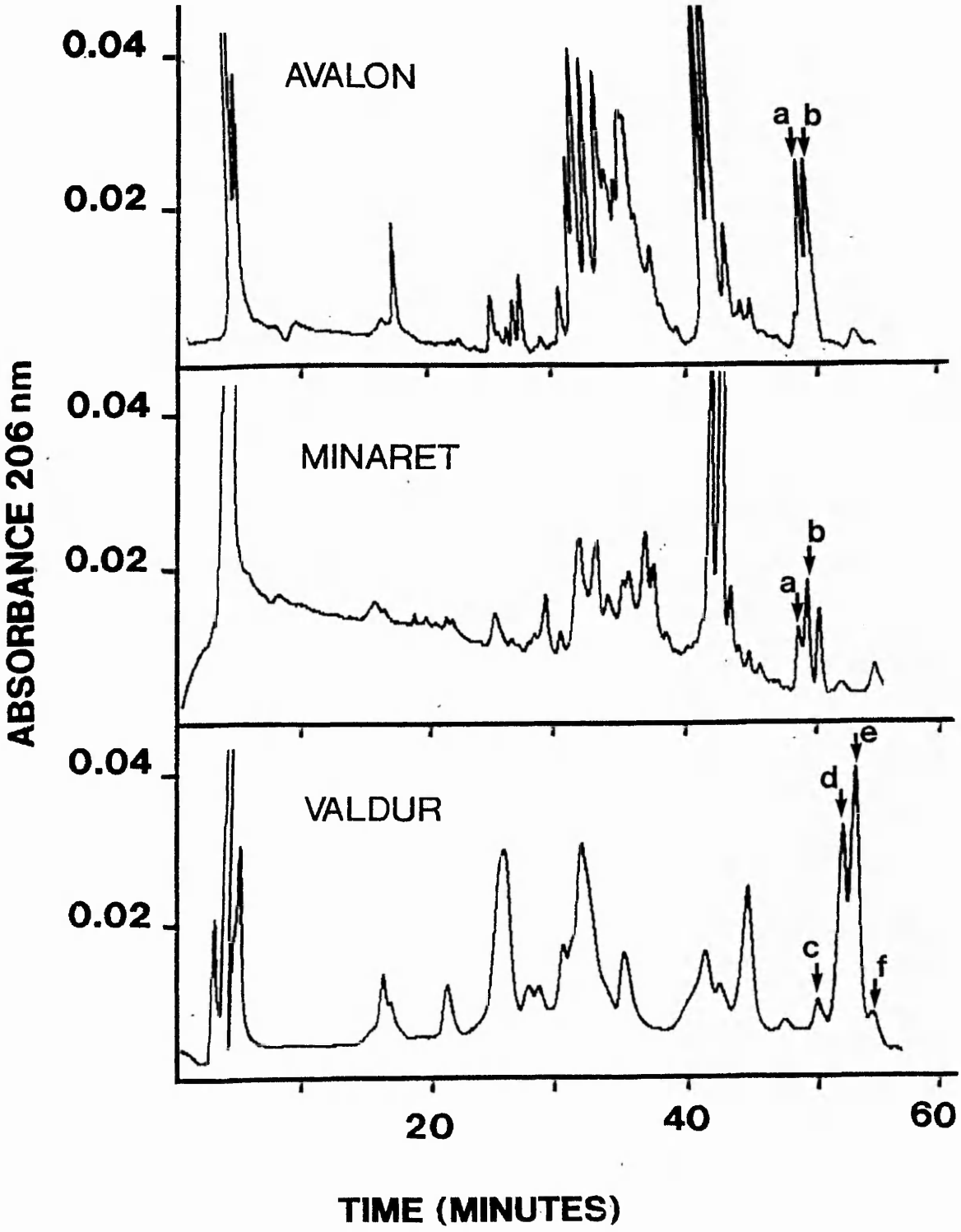
3.1.3.1. Comparison of Gliadin RP-HPLC Profiles of Common and Durum Wheat

Wheat gliadins from the hexaploid wheat varieties Avalon and Minaret and the Durum wheat variety D. Valdur were separated by RP-HPLC as shown in Figure 3.8. The resulting chromatograms were overlayed and examined very closely over the entire profile using the chromatogram reprocessing facilities of the Nelson Analytical Data Processor. Two major peaks eluted on the RP-HPLC profiles of Avalon (Figure 3.8, peaks a and b) and Minaret (Figure 3.8, peaks a and b) at 47.31 and 47.93 min. Corresponding peaks at this time were absent on the D. Valdur RP-HPLC profile. The gliadin RP-HPLC chromatograms from a further 36 varieties of European bred hexaploid wheats (Maris Dove, Crossbow, Rapier, Monzo, pernel, Sabre, Bounty, Slejpner, Parade, Riband, Tonic, Axona, Pastiche, Apollo, Mission, Sicco, Norman, Maris Huntsman, Alexandria, Brigand, Brimstone, Ferman, Jerico, Minaret, Kanzler, Huster, Galahad, Hobbit, Mercia, Rendezvous, Solitare, Brock, Broom, Hornet, Baron, Stetson), all had these two characteristic peaks eluting at 47.20+/-0.33 min and 47.94+/-0.05 min. The RP-HPLC profiles of 18 of these, have previously been shown in Figure 3.1. In some varieties of hexaploid wheat, (Bounty, Mission, Sicco), it was found that in addition to the peaks eluting at 47.20 min and 47.94 min two further major peaks eluted, at 50.30 and 52.46 min.

In the RP-HPLC profile of D. Valdur (Figure 3.8) no peak elution was observed at 47.20 min or 47.94 min. A small peak (Figure 3.8, peak c) eluted at 48.73 min on the D.

Figure 3.8. RP-HPLC Separation Profiles of Gliadin Extracts from Hexaploid and Durum wheat varieties.

Gliadin extracts (60μg of protein) were loaded onto a Water, Delta Pak, C18, 5μm particle size column (3.9x150 mm), and then eluted according to the procedure outlined in section 2.6. The profiles illustrated are Avalon, Minaret and Durum Valdur. Peaks at 47.31 min and 47.93 min (peaks a and b) are characteristic of Avalon and Minaret, peaks at 48.73 min, 50.36 min, 51.30 min and 52.80 min (peaks c,d,e,f) are characteristic of D. Valdur.


AVALON
MINARET
VALDUR
0.04
0.02
a
b
c
d
e
f
ABSORBANCE 206 nm
20
40
60
TIME (MINUTES)

Valdur gliadin profile which was not found in the hexaploid wheat profiles. Furthermore in the RP-HPLC profiles of D. Valdur two large peaks (Figure 3.8, peaks d and e) eluted at 50.36 and 51.30 min. Further varieties (Figure 3.9) of European bred Durum wheat (Capdur, Arcour, Flodur, Casoar, Regal, Primadur, Ambral, Mondur, Neodur, Durelle and Ardente) examined under the same conditions all had a small peak eluting at 48.83+/-0.08 min and two larger peaks eluting at 50.47+/-0.10 min and 51.37+/-0.07 min. A further small peak also eluted at 52.80+/-0.07 min in some varieties (Figure 3.8 peak f). This characteristic pattern of peak elution was also found in the RP-HPLC profiles of the semolina admixtures of French, British and Belgian Durum wheat, obtained from the LGC laboratories (Figure 3.9) and in the RP-HPLC profiles of the North American Durum wheat varieties (Edmore, Quilafen, Lloyd, Renville, Ward and Vic).

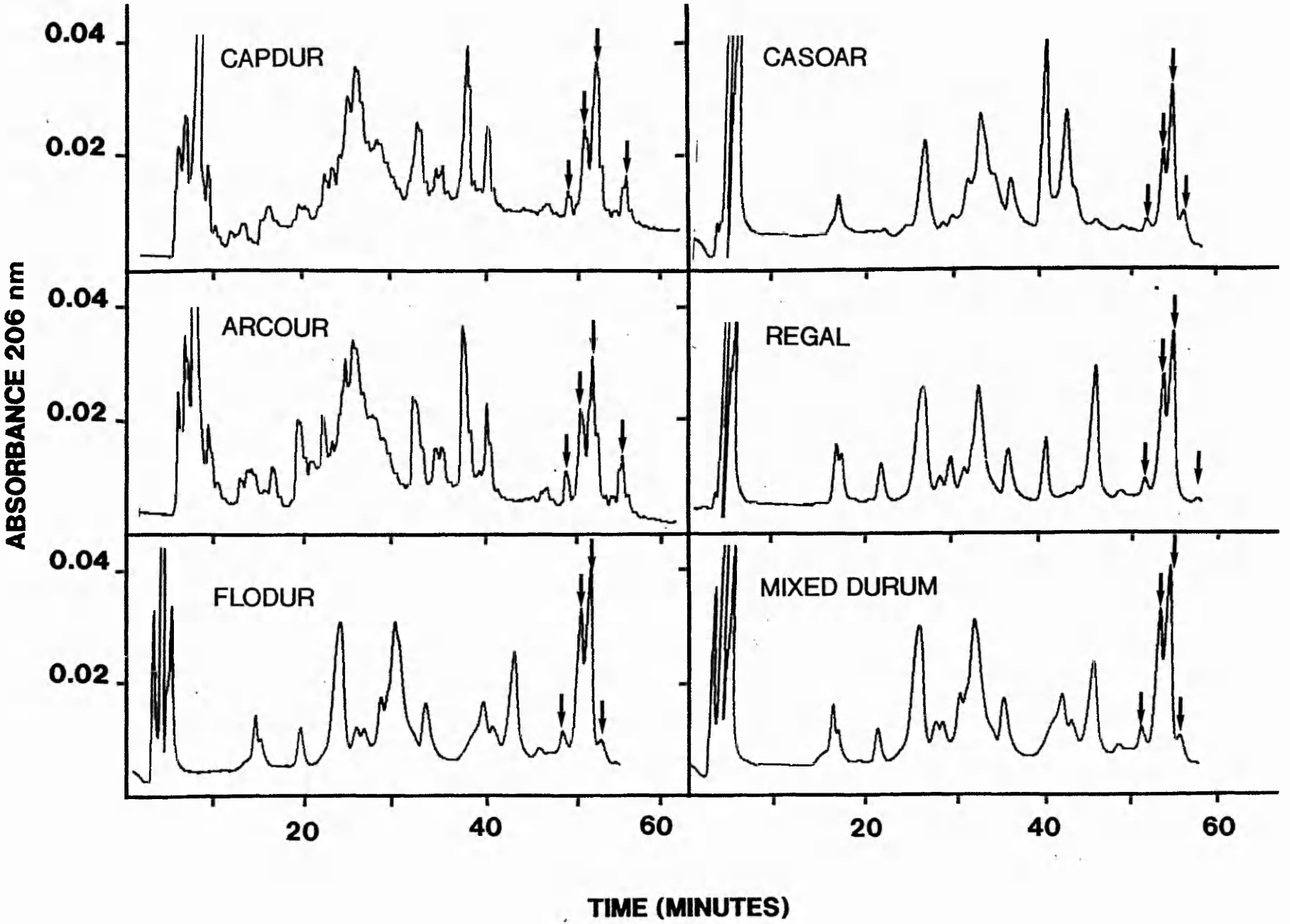
Similar analysis carried out on the above Durum and Hexaploid wheat, using the Waters Delta Pak, 10μm particle size column (4.6x300mm, C18, 300 Å), gave identical gliadin profiles. Peak retention times varied slightly. The peaks eluting at 47.20, 47.94, 48.83, 50.47, 51.37 and 52.80 min on the Waters 5μm particle size column (3.9x150mm) eluted at 48.90+/-1.04, 49.50+/-1.09, 49.75+/-1.10, 51.40+/-1.04, 52.40+/-1.21 and 54.70+/-1.21 min on the Waters 10μm particle size column (4.6x300mm).

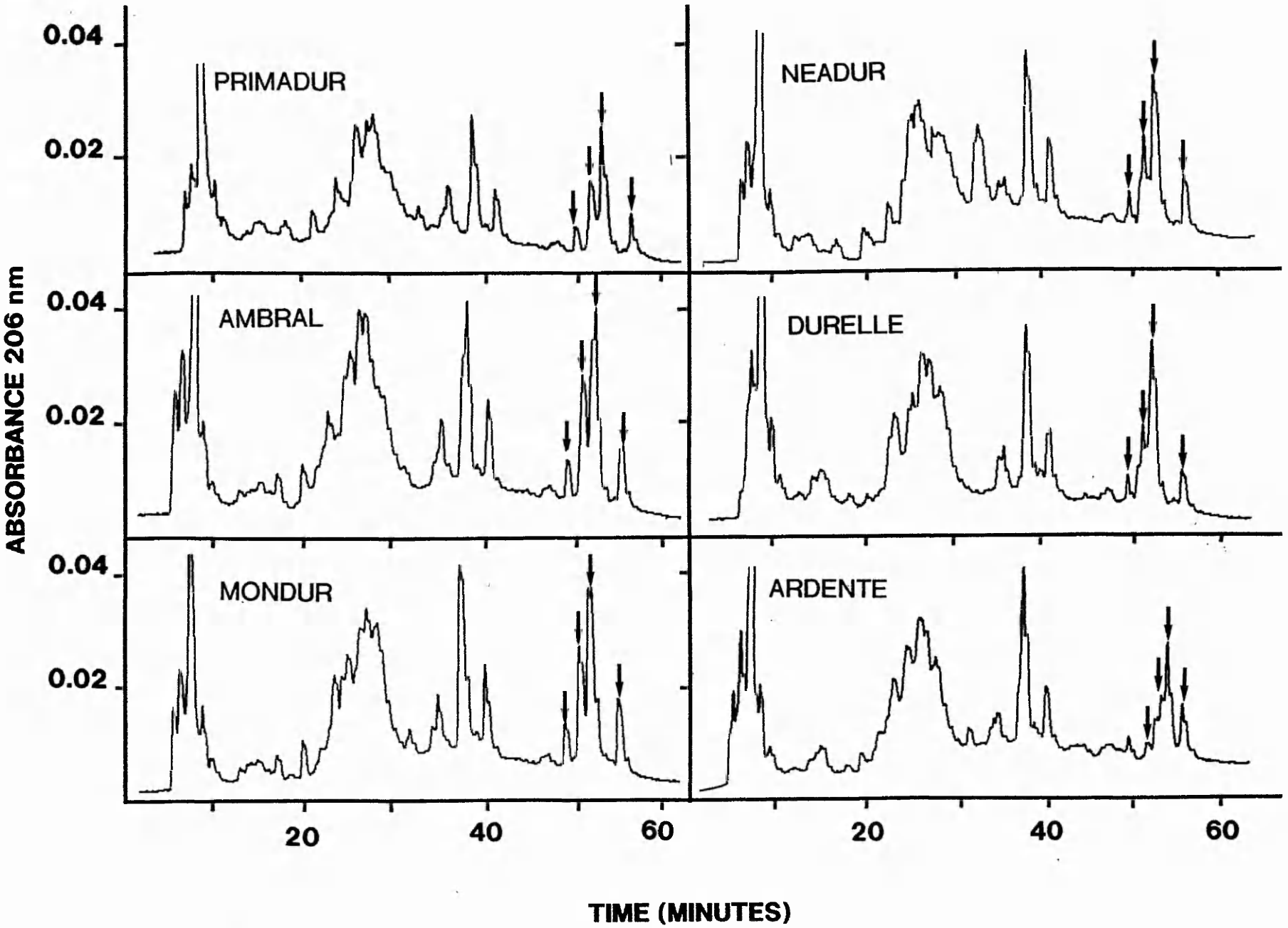
3.1.3.2. Electrophoresis of RP-HPLC Separated Gliadins

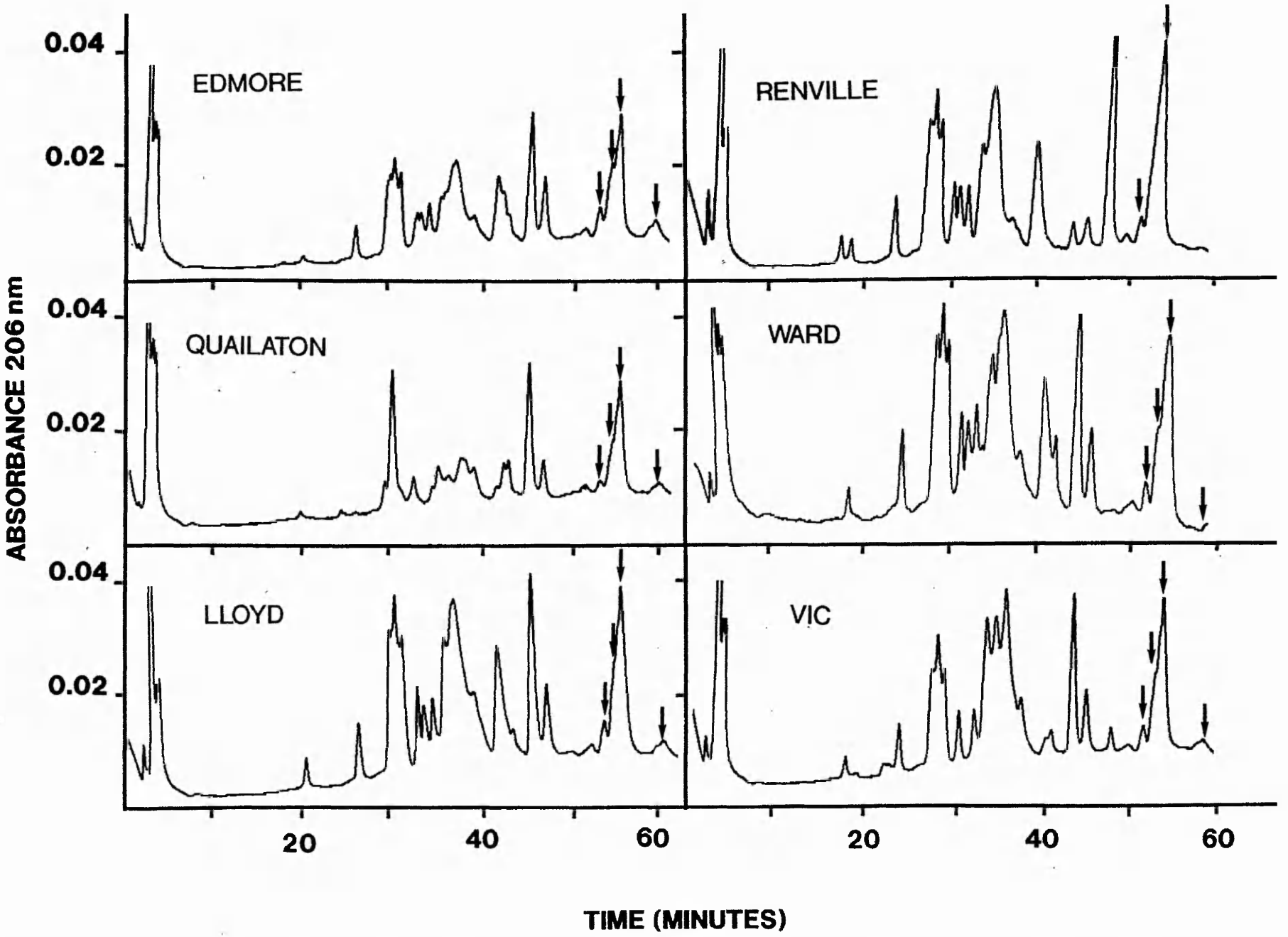
Fractions were collected between 0 and 60 min during the RP-HPLC separation of Avalon, and D. Valdur gliadins. Major consistent differences were observed on the

Figure 3.9 RP-HPLC Separation Profiles of Gliadin Extracts from Durum Wheat Varieties.

Gliadin extracts (60μg of protein) were loaded onto a Waters, Delta Pak, C18, 5μm particle size column, and then eluted according to the procedure outlined in section 2.6. The profiles illustrated are D. Capdur, D. Arcour, D. Flodur, D. Casoar, D. Regal, mixed Durum, D. Primadur, D. Ambral, D. Mondur, D. Neadur, D. Durelle, D. Ardente, D. Edmore, D. Quailaton, D. Lloyd, D. Renville, D. Ward, D. Vic arrows illustrate the characteristic peaks eluting at 48.83, 50.47, 51.37 and 52.80 min.


CAPDUR
CASOAR
ARCOUR
REGAL
FLODUR
MIXED DURUM
0.04
0.02
20
40
60
ABSORBANCE 206 nm
TIME (MINUTES)


PRIMADUR
NEADUR
AMBRAL
DURELLE
MONDUR
ARDENTE
0.04
0.02
ABSORBANCE 206 nm
20
40
60
TIME (MINUTES)


EDMORE
RENVILLE
QUAILATON
WARD
LLOYD
VIC
0.04
0.02
0.04
0.02
0.04
0.02
20
40
60
20
40
60
ABSORBANCE 206 nm
TIME (MINUTES)

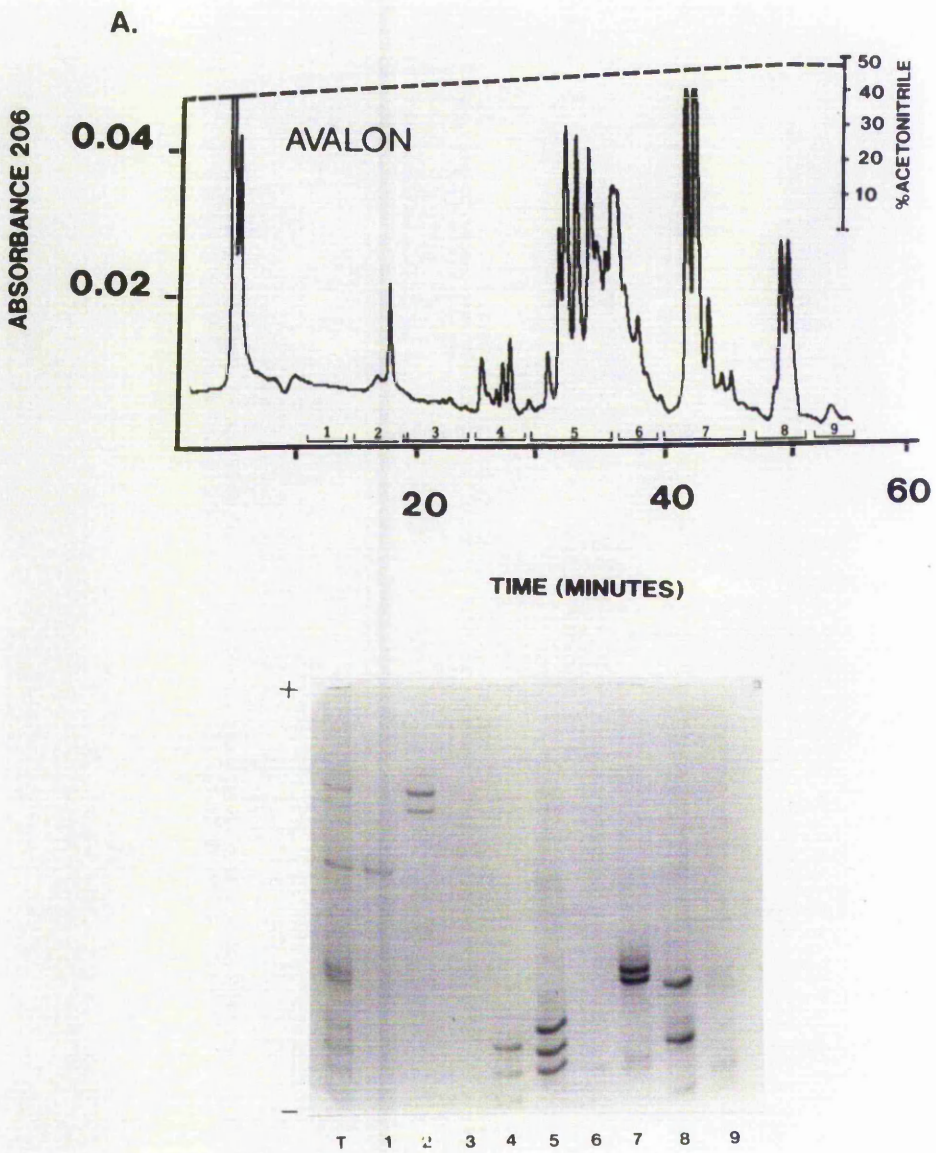
RP-HPLC profiles between 15-20, 47-49, 50-53 min (Figure 3.10 A and B). These differences were confirmed by gliadin electrophoresis using larger fractions collected using a Waters Delta Pak, C18, 10μm particle size column. To avoid confusion, retention times used to identify peaks eluting from the 5μm particle size column will continue to be used to identify peaks eluting from the 10μm particle size column.

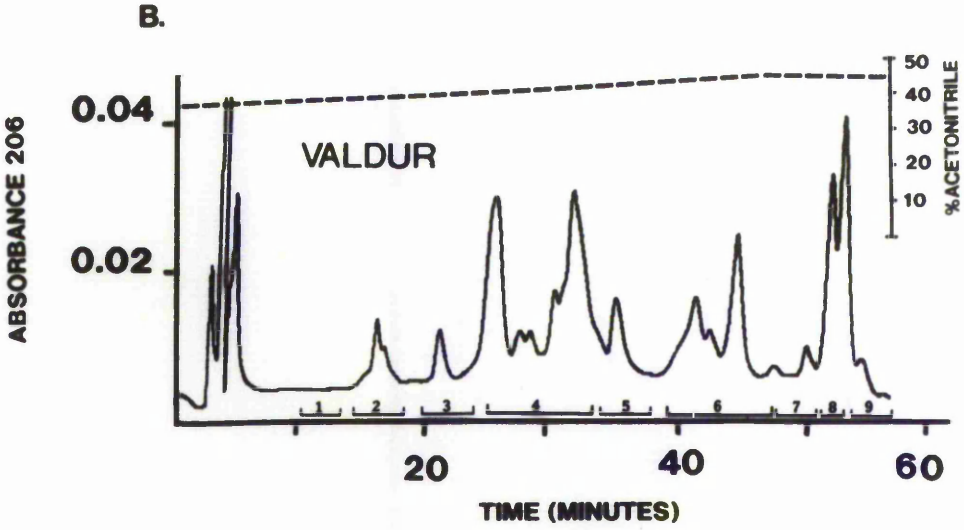
Two peaks (one major and one minor) were observed between 15-20 min on the gliadin RP-HPLC profile of Avalon (Figure 3.10A) and D. Valdur (Figure 3.10B). However from the Figure it is clear that the peaks from the different wheats have different retention times and thus represent different proteins. Gliadin electrophoresis (Figure 3.10) reveals that ω-gliadins are the main components eluting at this time. For both varieties early eluting ω-gliadins (common to both species) elute between 15-17 min (ie. the first peak) and late eluting ω-gliadins elute between 17-20 min (ie. the second peak). Late eluting ω-gliadins found only in hexaploid wheat varieties (section 1.2.1.4.) have been shown to be directly controlled by the 1D-genome (Metakovsky et al., 1984). The presence of these gliadins in Durum wheat is indicative of adulteration with common wheat. On RP-HPLC these gliadins elute between 17-20 min (Figure 3.10A). On electrophoretic patterns they are easily distinguishable from Durum wheat ω-gliadins by their slow mobility in comparison with Durum wheat ω-gliadins.

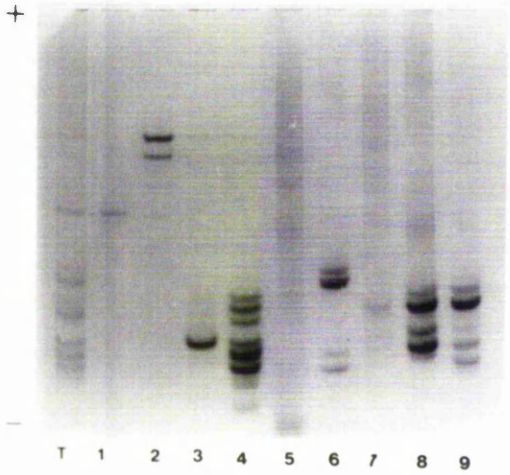
Electrophoresis of the Avalon fraction collected between 46.00 and 49.00 min showed that it consisted of 8 bands of γ/β-gliadins (Figure 3.10A). The latter 2 bands

Figure 3.10. Electrophoretic Separation Patterns of Gliadins collected during RP-HPLC Separation of Gliadin Extracts.

The chromatographic separations were carried out under the conditions outlined in section 2.4.1. and section 2.6. The profiles illustrated represent (A) Avalon and (B) Durum Valdur. Lanes 1-9 on the gel represent the gliadin electrophoretic patterns of chromatography fractions 1-9. Lane T represents the gliadin electrophoretic pattern of total extracts of Avalon and D. Valdur respectively.


A.
ABSORBANCE 206
0.04
0.02
AVALON
50
40
30
20
10
%ACETONITRILE
1
2
3
4
5
6
7
8
9
20
40
60
TIME (MINUTES)
+
−
T
1
2
3
4
5
6
7
8
9


B.
ABSORBANCE 206
0.04
0.02
VALDUR
50
40
30
20
10
%ACETONITRILE
1
2
3
4
5
6
7
8
9
20
40
60
TIME (MINUTES)


+
−
T
1
2
3
4
5
6
7
8
9

were also found in the fraction eluting between 50-53 min (Figure 3.10A) and were therefore not considered specific to this RP-HPLC fraction.

Electrophoresis of the D. Valdur fraction collected between 47.00 and 49.00 min (Figure 3.10B), showed that it consisted of 1 band of β-gliadin. Thus electrophoresis indicated that Avalon and Durum have only 1 common protein out of a possible total of 6 eluting from the reverse phase column between 47.00 and 49.00 min. Electrophoresis of the D. Valdur fraction collected between 50.00 and 52.00 min and 53.00 and 55.00 min (Figure 3.10B) showed that it consisted of 6 bands of γ/ β-gliadins. No protein bands were found in this fraction, which had eluted in the previous fraction (47-49 min). Electrophoresis of the Avalon fraction collected between 50.00 and 53.00 min showed that it consisted of 1 band of β-gliadin (Figure 3.10A).

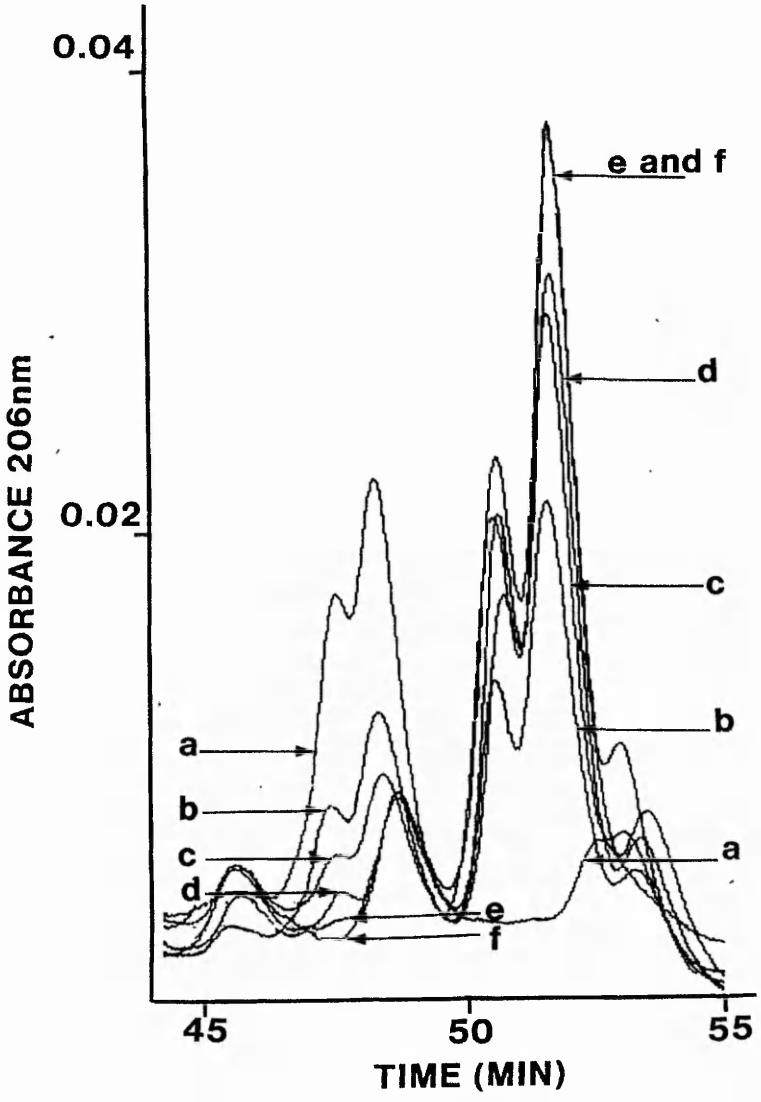
3.1.3.3. Detection of Common Wheat Adulteration of Durum Wheat Using RP-HPLC

i) Detection of Adulteration Using Peak Area/Protein Loading Ratio

The differences between the gliadin RP-HPLC elution profiles obtained from hexaploid wheat and Durum wheat species were further investigated as a basis for detecting and estimating the adulteration of Durum wheat flour with flour from hexaploid wheat varieties. The method developed involved separating a gliadin extract (of known protein concentration) of the suspect sample, using RP-HPLC (Figures 3.8, 3.11). The region of the elution profile between 45.00 and 55.00 min was examined for the presence of

Figure 3.11. RP-HPLC Separation Profiles of Durum Gliadin Extracts containing (0-50%) Hexaploid Wheat.

Gliadin extracts (60μg of protein) were loaded onto a Waters, Delta Pak, C18, 5μm particle size column, and then eluted according to the procedure outlined in section 2.6. The profiles illustrated in overlay are; (a), 100% Avalon, (b), 50% Avalon in D. Valdur, (c), 20% Avalon in D. Valdur, (d), 10% Avalon in D. Valdur, (e), 5% Avalon in D. Valdur, (f), 100% D. Valdur.


0.04
0.02
ABSORBANCE 206nm
e and f
d
c
b
a
a
b
c
d
e
f
45
50
55
TIME (MIN)

peaks. If the applied sample contains only Durum gliadins, 4 peaks should be detected in this area eluting at 48.83, 50.47, 51.37, and 52.80 min (Figure 3.8). If the sample is adulterated, further peaks elute between 47.00 and 48.00 min (Figure 3.11). If the level of adulteration is severe (>15%) the peak normally found at 48.83 becomes distorted and can then cover the whole area between 47.00 and 49.00 min as a doublet peak. By expressing the entire integrated area eluting between 47.00 and 49.00 min as a ratio with the amount of protein applied to the column, a value is obtained which is found to be the maximum permitted value in a sample claiming to be pure Durum wheat. The actual ratio values obtained for 6 Durum varieties and 2 semolina flours are shown on Table 3.5. Statistically these values were found to have a mean value of 561 +/- 61, and a 95% confidence interval between 513-620. Thus an adulterated sample with a level of adulteration greater than 5 per cent (Table 3.5B, ratio value = 602 +/-24) may be detected when the peak area/protein applied ratio is compared to that of the Durum standards (Table 3.5A).

To demonstrate this hypothesis, gliadins were extracted from mixtures of D. Valdur adulterated with Avalon, at levels of 5%, 10%, 15%, 20%, 30%, 50% and the extracts were separated by RP-HPLC. The resulting profiles are shown in an overlaid form in Figure 3.11. As the level of adulteration increased a doublet peak emerges between 47.00 and 49.00, the apparent size of this peak increased as the level of adulteration increased. Adulteration at levels as low as 5% was clearly visible. Comparison of this, with the

Table 3.5. Relationship between area of peaks eluting between 47 - 49 min and total protein applied to a RP-HPLC column

WHEAT VARIETY	AREA OF PEAKS (a+b+c) PROTEIN LOADING (μg) (n = 3) MEAN +/- SD	
A		
D. Capdur	558 (+/- 17)	
D. Flodur	592 (+/- 1)	
D. Valdur	580 (+/- 20)	
D. Casoar	462 (+/- 27)	
D. Arcour	480 (+/- 7)	
D. Regal	570 (+/- 40)	
Semolina 1	615 (+/- 31	
Semolina 2	633 (+/- 33)	
Mean +/- SD	561 (+/- 61)	
95% CI	513 - 620	
B		
D. Valdur + % Adulteration		p-Values
0	580 (+/- 20)	
5	602 (+/- 24)	NS*
10	655 (+/- 30)	p < 0.050
15	964 (+/- 11)	p < 0.005
20	1161 (+/- 59)	p < 0.005
30	2783 (+/-194)	p < 0.005
40	4053 (+/-157)	p < 0.005
Correlation co-efficient of % adulteration and ratio = +0.97		

*values not statistically significant.

A. Gliadins extracted from flour from different Durum wheat
B. D. Valdur adulterated with varying levels (5%, 10%, 15%, 20%, 30%, 50%) of a common wheat admixture (25% each of Avalon, Moulin, Maris Huntsman, Hobbit). Ratios are calculated from protein loadings varying between 50 - 70 μg of protein, (as outlined in the method section). Data represents the mean of three experiments.

gliadin electrophoretic pattern of the same samples (Figure 3.12), clearly highlighted adulteration by the presence of the characteristic slow eluting ω-gliadins at 20% and 50% adulteration, at 10% these bands were barely detectable and it was expected from this test that adulteration levels less than 20% would be difficult to detect using electrophoresis alone.

A standard curve (Figure 3.13) was constructed relating the ratio of the peak (47.00 - 49.00 min) area / protein applied (Table 3.5B, correlation coefficient = 0.97), calculated for each Durum/hexaploid wheat admixture at the different levels (0-30%) of adulteration. As adulteration is usually caused by mixtures of hexaploid wheat rather than a single variety, the hexaploid wheat mixture chosen for use as adulterant was a blend containing equal concentrations of Avalon, Moulin, Maris Huntsman and Hobbit. The peak area ratio values of unknown wheats could be compared to this curve in a manner similar to a normal standard curve and a quantitive estimate was obtained for levels of adulteration greater than 10 percent (Table 3.5B, $p < 0.05$). Visual detection was possible as low as 5 percent (Figure 3.11).

ii) Adulteration Detection Using Electrophoresis Following RP-HPLC

By combining RP-HPLC and PAGE, a further method was used for the detection of adulteration of Durum wheat with hexaploid wheat varieties, if further confirmation of adulteration was required. Having separated the gliadin extract using RP-HPLC, fractions corresponding to the area between 17.00 and 20.00 min and 47.00 and 49.00 min were

Figure 3.12 Electrophoretic Separation Pattern of Gliadin Extracts from Durum and Common Wheat Blends.

Electrophoresis was carried out according to the method outlined in section 2.4.1. The patterns illustrated represent (1), 100% Avalon, (2), 50% Avalon in D. Valdur, (3), 20% Avalon in D. Valdur, (4), 10% Avalon in D. Valdur, (5), 5% Avalon in D. Valdur, (6), 100% D. Valdur.

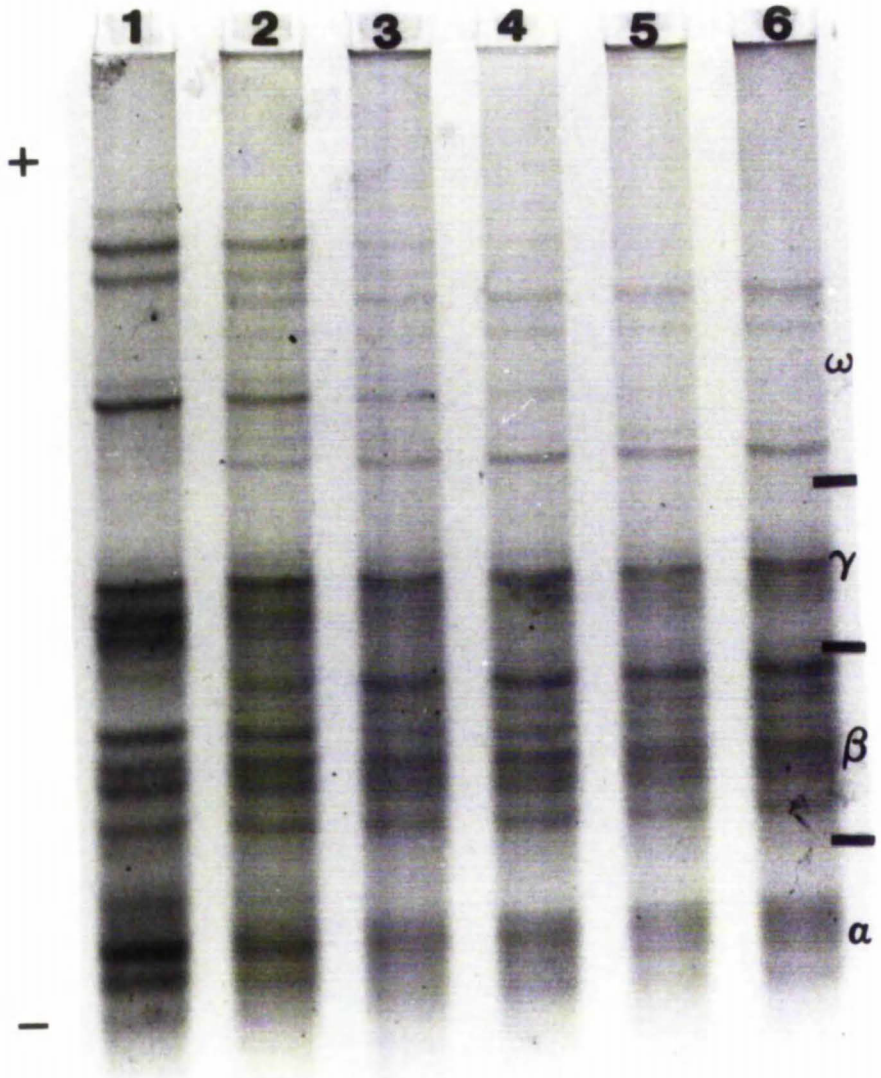
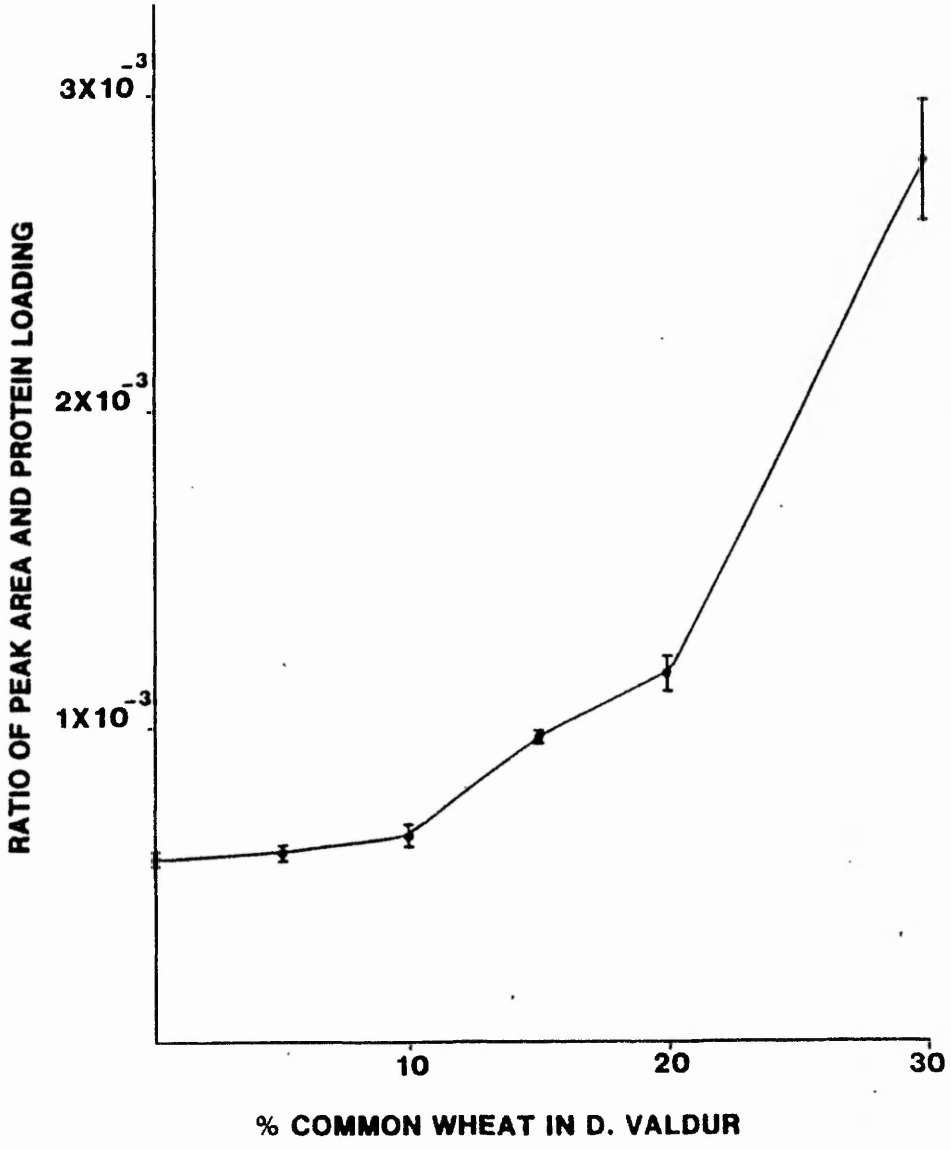

1
2
3
4
5
6
+
−
ω
γ
β
α

Figure 3.13. Standard Curve relating Peak Area Ratio with the Level of Hexaploid wheat in a Durum Wheat Sample

Extracts from pure D. Valdur and D. Valdur after adulteration (0-30%) with a hexaploid wheat mixture (containing the varieties Avalon, Moulin, Hobbit and Maris Huntsman) were separated by RP-HPLC according to the method outlined in section 2.6.


RATIO OF PEAK AREA AND PROTEIN LOADING
3X10⁻³
2X10⁻³
1X10⁻³
10
20
30
% COMMON WHEAT IN D. VALDUR

collected and concentrated by freeze drying thereby facilitating further analysis of the protein using acid electrophoresis.

If ω-gliadins characteristic of Durum wheat and one band of β-gliadin were identified on the electrophoregram representing the fractions eluting between 17-20 min and 47-49 min respectively, the sample was considered to be pure Durum wheat. If however hexaploid wheat specific ω-gliadins and more than one band of γ/β-gliadins were identified on the electrophoregrams representing the fractions eluting between 17-20 min and 47-49 min respectively then the sample was considered to be adulterated with hexaploid wheat. Although it may not be possible to quantify the exact level of adulteration by this method, it was shown, after repeated collection of the protein eluting between 17-20 min and 47 - 49 min that subsequent electrophoresis could detect very low levels of adulteration (even 1% adulteration could be detected). However the use of this method is limited by the fact that the procedure is long and complicated and requires a great deal of technical input.

3.1.3.4 Discussion

When 100% pure Durum wheat gliadins were separated under the conditions described a characteristic small peak elutes at 48.83+/-0.08 min, (Figure 3.8, peak c), followed at 50.47+/-0.10 min, 51.37+/-0.07 min and 52.80+/-0.07 min by larger characteristic peaks (Figure 3.8, peaks d, e, f). In comparison when hexaploid wheat gliadins were separated under identical conditions a major doublet peak elutes at 47.20+/-0.33 min and 47.94+/-0.05 min, (Figure 3.8, peaks a and b

respectively). These differences were used as the basis of a method to detect the level of adulteration of Durum wheat by hexaploid wheat.

The ratio of the integrated peak area between 47.00 - 49.00 min and total protein extract applied in a pure Durum wheat sample was found to be 561+/-61 with a 95% confidence interval between 513 - 620 (Table 3.5). Samples with levels of adulteration greater than 5 per cent were detected and by reference to a standard curve (Figure 3.13) the adulteration, when greater than 10 per cent could be quantified when the peak area / protein applied ratio of the suspect gliadins were compared with the values obtained for peak area ratios of the standard admixtures of Durum and hexaploid wheat. The values (Table 3.5) used to construct this standard curve were the mean values taken from D. Valdur samples adulterated with a sample of hexaploid wheat containing Avalon, Moulin, Maris Huntsman and Hobbit. Using admixtures reduces the error associated with the genetic diversity of hexaploid wheats which causes differences in the area of the peaks eluting between 47.00 and 49.00 min in hexaploid wheats. Furthermore Durum wheat adulteration is usually caused by a variety of different hexaploid wheats and again a standard admixture containing more than one variety of hexaploid wheat will give a more accurate representation of the level of adulteration.

It has been shown by Cooke *et al.* (1986) and Metakovsky *et al.* (1984) that the two wheat species can be distinguished from one another using acid gel electrophoresis since Durum wheat lacks the prolamins encoded by the D-

genome, most noticeably the ω-prolamin type believed to be controlled by genes on chromosome 1D. It was proposed by Kobrehel et al. (1985) that this difference could be used as the basis for a test to detect adulteration of Durum wheat flour with hexaploid wheat flour but no indication of the sensitivity of this method was given. In this study it has been shown that PAGE of total gliadin proteins at low pH values can detect adulteration of Durum wheat flour by common wheat flour at levels as low as 10% (Figure 3.12). However the subjective nature of this method means that it is not ideal for detecting adulteration at levels less than 20%.

The combination of RP-HPLC and acid PAGE allows concentration of the adulterating gliadins in a pasta sample and gives confirmation of a result from either technique. It has previously been demonstrated by Cooke et al. (1986), using electrophoresis that along with the differences shown between hexaploid and Durum wheats in the ω-gliadin region of the gel there are also some characteristic differences between the two species in the γ/β-regions of the gel. It is suspected therefore that the proteins eluting between 47.00 and 49.00 min following RP-HPLC of hexaploid wheat gliadins (as outlined on Figure 3.8), are in fact proteins encoded by the D-genome.

In conclusion to this section it can be stated that RP-HPLC separation of 25% v/v 2-chloroethanol extractable gliadins proved a reliable way of detecting and quantifying adulteration of Durum wheat with common wheat flour. A large number of the wheat varieties used in this study were of UK origin. But the studies using Durum wheat

obtained from North America and France and semolina meal obtained from Belgium and France, combined with hexaploid wheat from Germany, Sweden, Holland and France suggest that the method may have the potential to be used much wider than the UK and the European community. Its main advantages being its increased sensitivity and its ability to produce a quantitative result within 90 min of receipt of sample. Acid PAGE of gliadins requires up to 5h and TLC of fatty acid steroid can take up to 3 days. The application of this method to heated semolina products and its comparison with existing methods is presented in the following section.

3.2. EVALUATION OF RP-HPLC TO DETECT Triticum Aestivum IN PASTA PRODUCTS DRIED AT HIGH TEMPERATURE; A COMPARISON WITH EXISTING METHODOLOGY.

To comply with national legislation pasta manufacturers in Italy can only prepare their produce from 100 per cent Durum wheat (Triticum Durum) and manufacturers in the UK and other EC countries are similarily constrained by national food labelling regulations if they declare their products to be manufactured from "100 percent Durum wheat". However it has become apparent (personal communication Mr I Lumley, LGC) that pasta products labelled as being prepared from Durum wheat only and sold in many major UK outlets contain undeclared quantities of flour from common wheat varieties (Triticum aestivium). In order to enforce food labelling regulations and to ensure correct tariff classification of imported pasta products it is important that EC legislation enforcement authorities and custom laboratories have access to reliable analytical methodology capable of detecting and determining common wheat flour in Durum wheat pasta products declared as containing 100% Durum wheat.

Many different techniques (as outlined in section 1.1.6.3.) are used to detect common wheat adulteration of Durum wheat. However many of these are based on determining differences in wheat components which are often heat labile thereby making their application to pasta products highly subjective. In addition pasta production technology increasingly uses temperatures in excess of 90^{o}C in the manufacture and drying of pasta. This further limits the use

of many of the adulteration detection methods. To date no comparative study has been made of the different methods available to detect common wheat adulteration of pasta nor has the effect of high temperature processing on these methods been assessed.

In this study the RP-HPLC technique previously developed (section 3.1.3) has been applied to the detection of common wheat adulteration of pasta dried at elevated temperature. Furthermore this method is compared to the more established methods, with a view to assessing its suitability for the detection of common wheat adulteration of pasta products dried in a superheat drying cycle.

3.2.1. Effects Of Drying Temperature on Detection Methods

Comparisons were made between two batches of commercial Durum wheat, semolina flour and pasta dried at both 40°C and 85°C. No significant difference between unmilled Durum wheat and its semolina flour was apparent after tlc analysis of fatty acid sterol ester extracts (Figure 3.17), electrophoretic analysis of various protein extracts (Figures 3.14-3.16) and RP-HPLC separation of gliadin extracts (Figure 3.18). In all further comparisons results are only reported for pasta and its semolina flour as it is assumed that any differences attributable to high drying temperatures observed between a pasta sample and its original semolina flour are also observed between the pasta sample and its unmilled wheat flour.

Drying at temperatures up to 40°C caused no observable change in the polyphenoloxidase activity of pasta, as measured by catechol activity, when compared to that of semolina flour by alkaline PAGE of water soluble extracts of pasta and semolina flour (Figure 3.14A lanes 3, 2 respectively). Contrary to this the polyphenoloxidase activity observed in electrophoregrams of pasta dried at high temperature (ie 85°C) was severly reduced (Figure 3.14B, lane 3) when compared to the activity detected in its semolina flour (Figure 3.14B, lane 2) and in pasta dried at 40°C (Figure 3.14A, lane 3). Similarily the gliadin electrophoretic pattern of pasta dried at 40°C showed no evidence of heat denaturation when compared to the gliadin electrophoresis pattern of semolina flour treated similarily (Figure 3.15A lanes 3, 2 respectively). However a

Figure 3.14. Alkaline PAGE Separation Patterns of Specific Durum Wheat Polyphenoloxidases

Samples were taken at different stages during the commercial manufacture of pasta dried at (A) 40°C and (B) 85°C. These samples illustrate (1) Durum wheat flour (2) Durum wheat semolina (3) dried pasta.

Electrophoresis was carried out according to the method outlined in section 2.4.2.

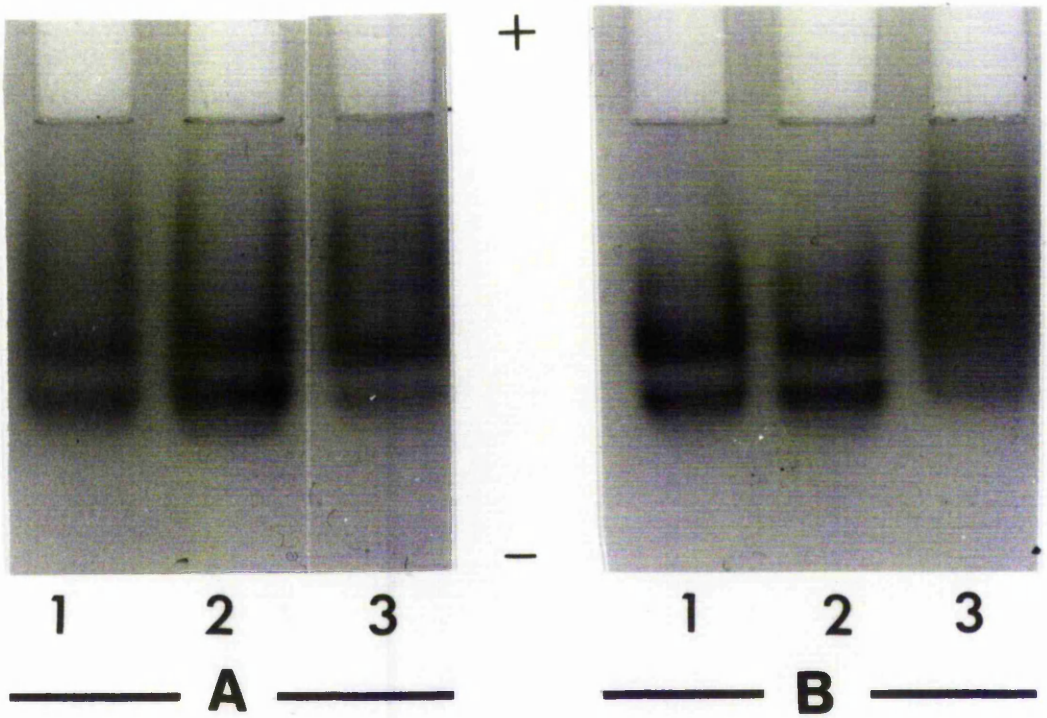
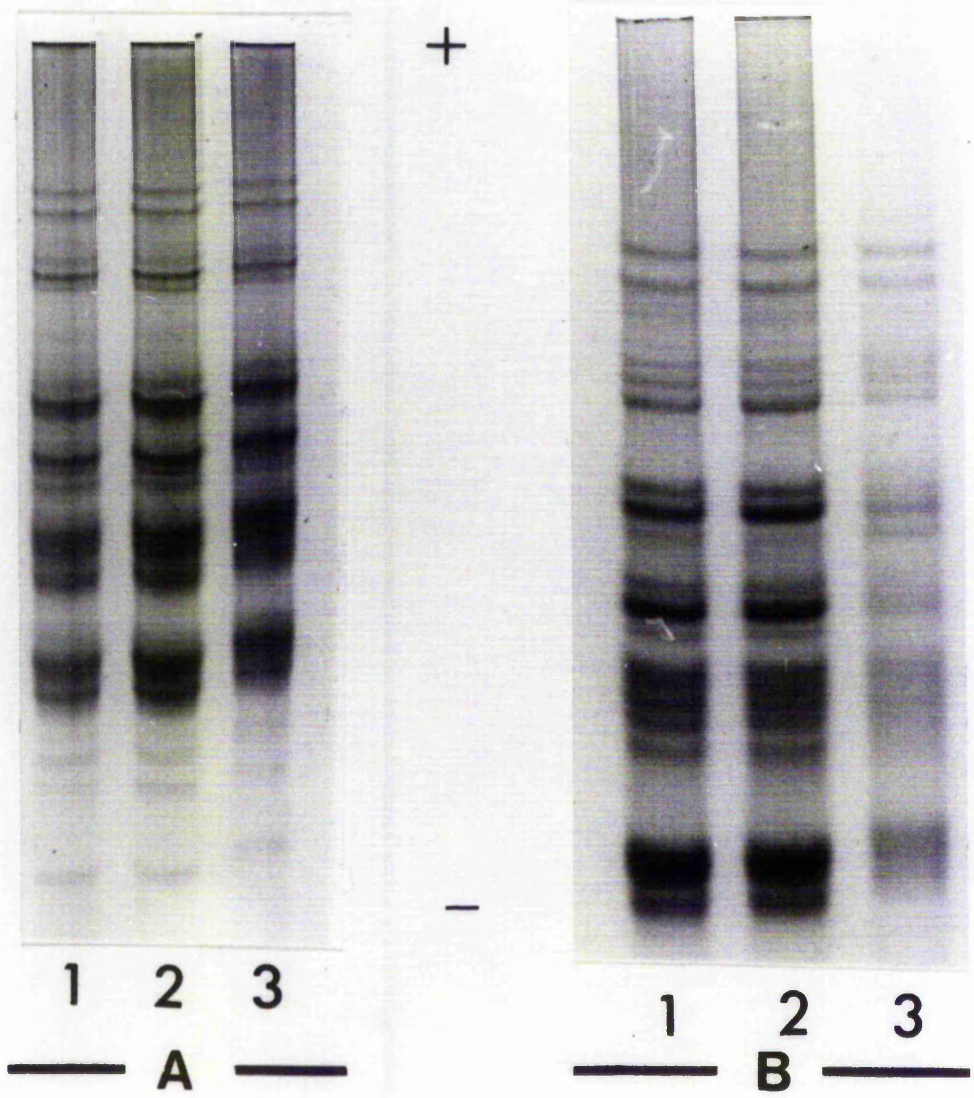

+
−
1
2
3
A
1
2
3
B

Figure 3.15. Acid PAGE Separation Patterns of Alcohol Soluble Durum Wheat Gliadins

Samples were taken at different stages during the commercial manufacture of pasta dried at (A) 40°C and (B) 85°C. These samples illustrate (1) Durum wheat flour (2) Durum wheat semolina (3) dried pasta.

Electrophoresis was carried out according to the method outlined in section 2.4.1.


+
−
1 2 3
A
1 2 3
B

significant loss of resolution was observed in the gliadin electrophoretic pattern of pasta dried at 85°C (Figure 3.15B lane 3). Using IEF the esterase composition of pasta dried at 40°C showed no evidence of heat damage, (Figure 3.16A lane 3) but total destruction of esterase activity occured in pasta dried at 85°C (Figure 3.16B lane 3) as indicated by the complete loss of esterase bands observed in the IEF electrophoregram of this sample compared to that of semolina flour (Figure 3.16B, lane 2). Loss of resolution and/or enzyme activity in each of the electrophoretic methods, usually attributable to protein denaturation, was therefore only observed in pasta samples exposed to high temperature drying conditions. This fact casts doubt on the ability of these analytical methods to detect the presence of common wheat in Durum wheat pasta products dried at elevated temperatures.

Drying at temperatures up to 85°C had no observable effect on the fatty acid sterol ester composition of pasta when petroleum ether extracts of pasta dried at 40°C (Figure 3.17A lane 3) and 85°C (Figure 3.17B lane 3) were compared with their respective semolina flours by tlc. This procedure may therefore be suitable for detection of common wheat adulteration of pasta dried at elevated temperatures.

The gliadin RP-HPLC profiles obtained from commercial pasta dried at 40°C, and that of the original semolina flour were also found to be similar (Figure 3.18A). Some peak distortion was observed throughout the gliadin RP-HPLC profile obtained from pasta dried at 85°C (Figure 3.18B) when compared to the gliadin RP-HPLC profile of the original

Figure 3.16. IEF Separation Patterns of Durum Wheat Water Soluble Esterases

Samples were taken at different stages during the commercial manufacture of pasta dried at (A) 40°C and (B) 85°C. These samples illustrate (1) Durum wheat flour (2) Durum wheat semolina (3) dried pasta.

IEF was carried out according to the method outlined in section 2.4.5.

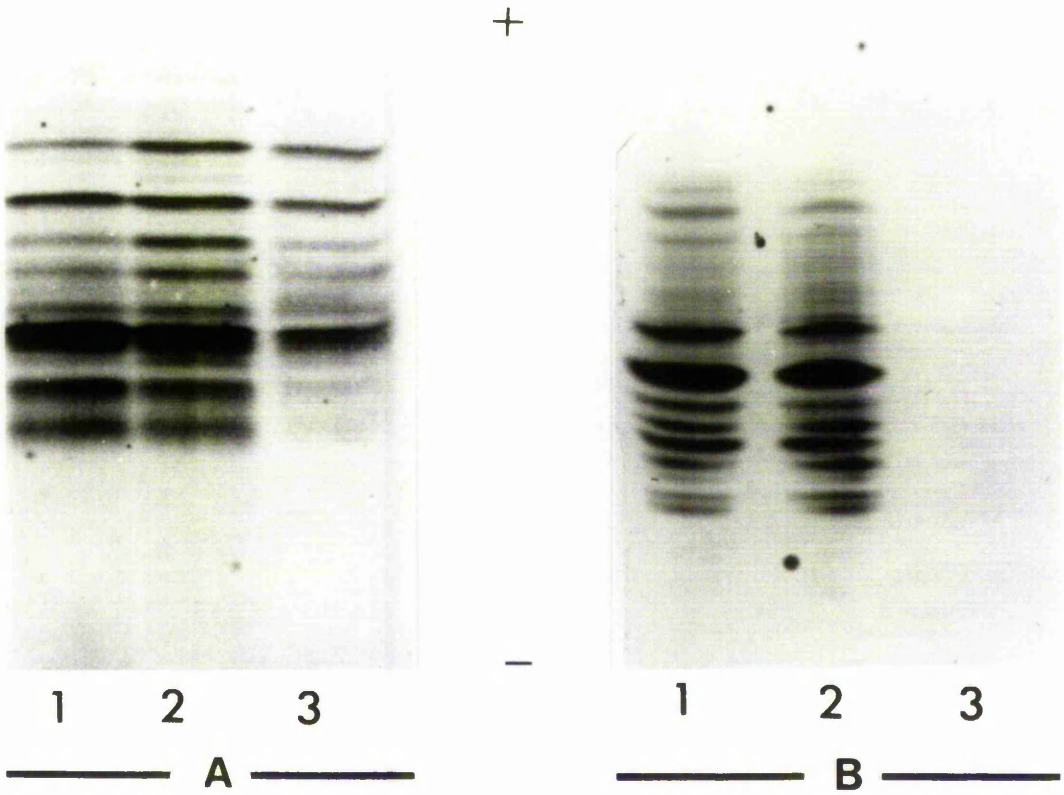

+
−
1
2
3
A
1
2
3
B

Figure 3.17. TLC Separation Patterns of Durum Wheat Petroleum Ether Soluble Fatty Acid Sterols

Samples were taken at different stages during the commercial manufacture of pasta dried at (A) 40°C and (B) 85°C. These samples illustrate (1) Durum wheat flour (2) Durum wheat semolina (3) dried pasta.

TLC was carried out according to the method outlined in section 2.5.

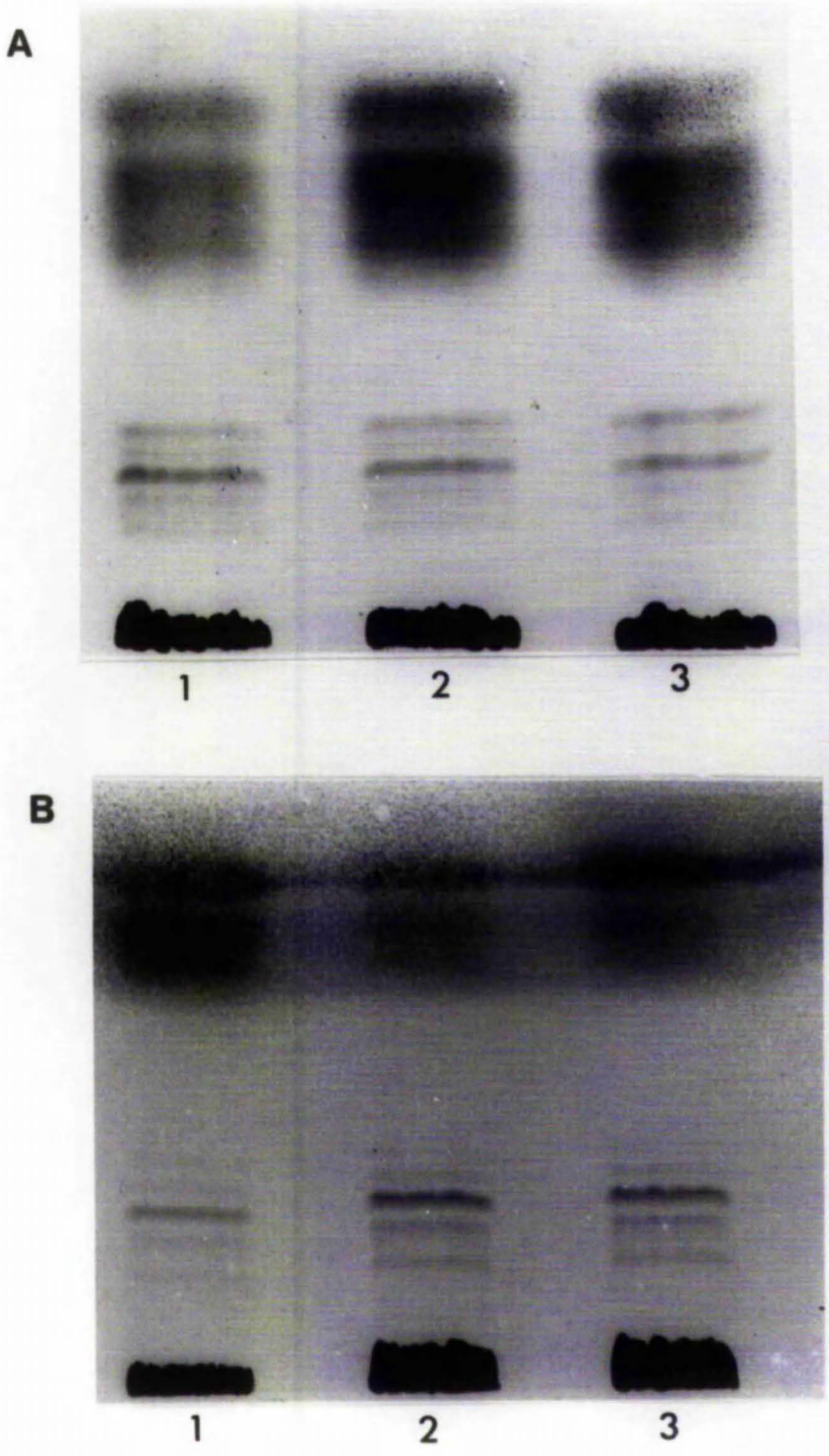
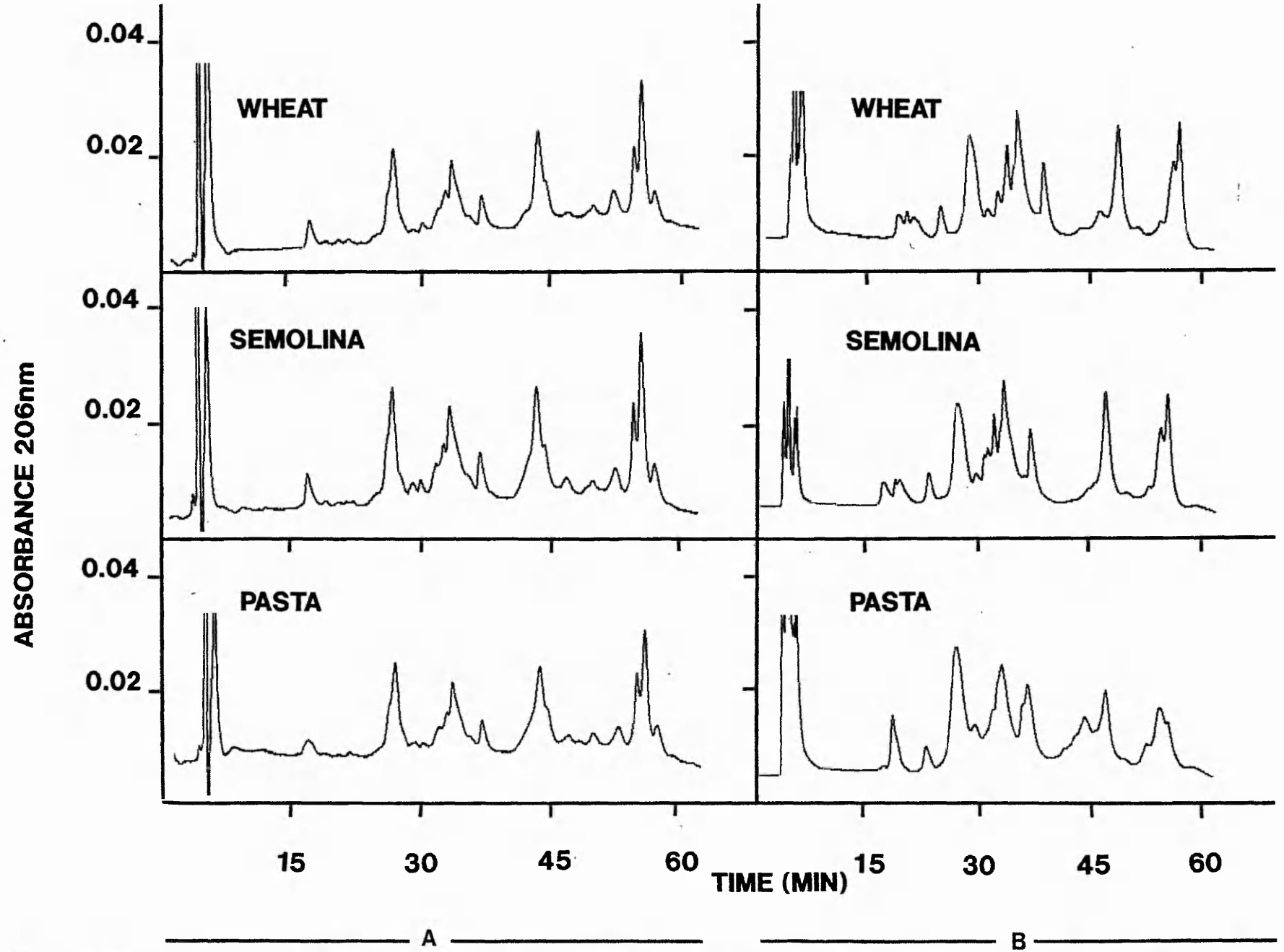

A
1
2
3
B
1
2
3

Figure 3.18. RP-HPLC Separation Profiles of Durum Wheat Alcohol Soluble Gliadins

Samples were taken at different stages during the commercial manufacture of pasta dried at (A) 40°C and (B) 85°C. These samples illustrate (1) Durum wheat flour (2) Durum wheat semolina (3) dried pasta. Gliadin extracts (60μg of protein) were loaded onto a Waters, Delta pak, C18, 5μm particle size column (3.9x 150mm) and eluted according to the method outlined in section 2.6.


0.04
0.02
WHEAT
WHEAT
0.04
0.02
SEMOLINA
SEMOLINA
0.04
0.02
PASTA
PASTA
ABSORBANCE 206nm
15
30
45
60
TIME (MIN)
15
30
45
60
A
B

semolina flour. Nevertheless the area of the chromatogram which was used to detect common wheat adulteration (peaks eluting between 47-49 min, indicated by arrows Figure 3.18) appeared unaffected by heat. This method was therefore capable of detecting the adulteration of pasta dried at high temperature.

3.2.2. Detection of Common Wheat Adulteration in Commercial Pasta Samples

A number of pasta samples declared to be produced from 100% Durum wheat but suspected of containing common wheat flour were analysed by the different procedures and the resulting data was compared and summarised in Table 3.6. The drying temperature of each of the samples was unknown.

Using the electrophoretic patterns of specific polyphenoloxidases a band of high mobility, not normally associated with Durum wheat (Figure 3.19, lane 2), was found in the electrophoretic patterns of three pasta samples (samples 1, 2 and 3, Figure 3.19 lanes 3-5, band a). It has been shown (Feillet and Kobrehel, 1974) that the presence of this protein band, (unique to common wheat, Figure 3.19, lane 1, band a), indicates that the pasta samples are adulterated with common wheat. However, two pasta samples treated similarily, (samples 4 and 5, Figure 3.19 lanes 6, 7) yielded patterns with a much reduced resolution making it difficult to determine if this protein band and hence common wheat was present.

The presence of esterases unique to common wheat (Figure 3.20, lane 2, bands b, c and d), were found in the esterase IEF patterns of three pasta samples

Table 3.6 Detection of Common Wheat Adulteration of Pasta Samples Using Different Detection Procedures

Pasta Sample	Methods Based on Detection of: Polyphenol-oxidases	Esterases	Sterol Esters	ω-Gliadins	RP-HPLC Peaks
1	+[a]	+	+	+	+
2	+	+	+	+	+
3	+	+	-	-	+
4	-[b]	-	-	-	-
5	-	-	-	-	-

[a] + indicates that common wheat adulteration is detected by this method

[b] - indicates that common wheat adulteration is not detected by this method

Figure 3.19. Alkaline PAGE Separation Patterns of Specific Polyphenoloxidases Extracted from Pasta Samples

The samples illustrated are as follows: lane 1, common wheat flour; lane 2, Durum wheat semolina; lanes 3-7 represent pasta samples 1-5 respectively.
Electrophoresis was carried out according to the method outlined in Figure 3.14.

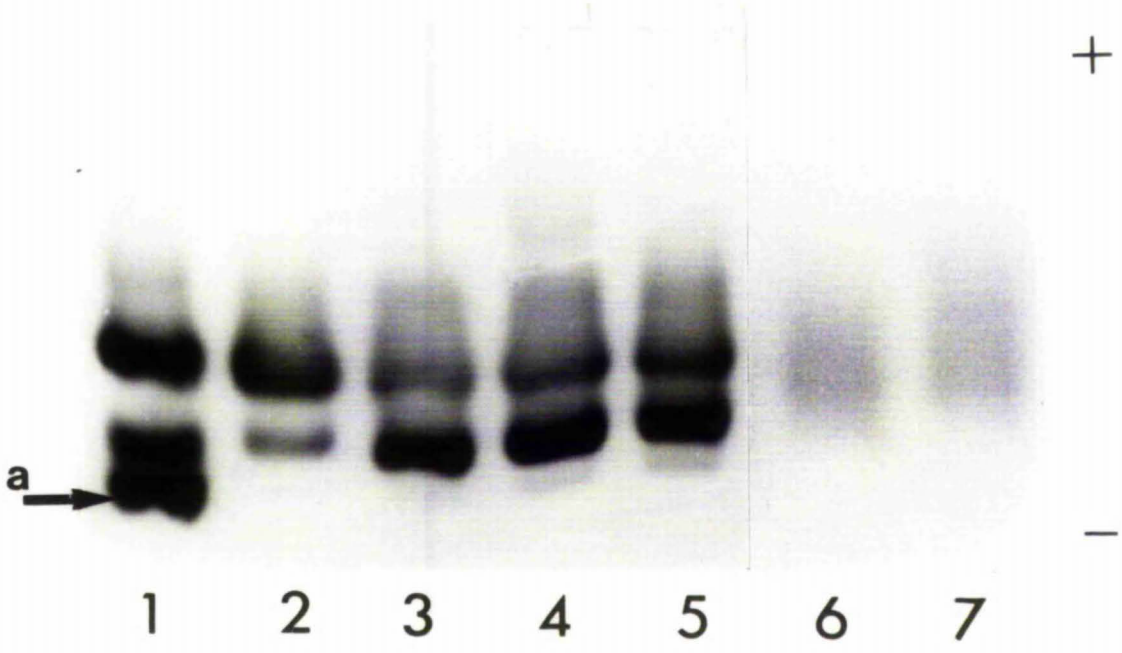
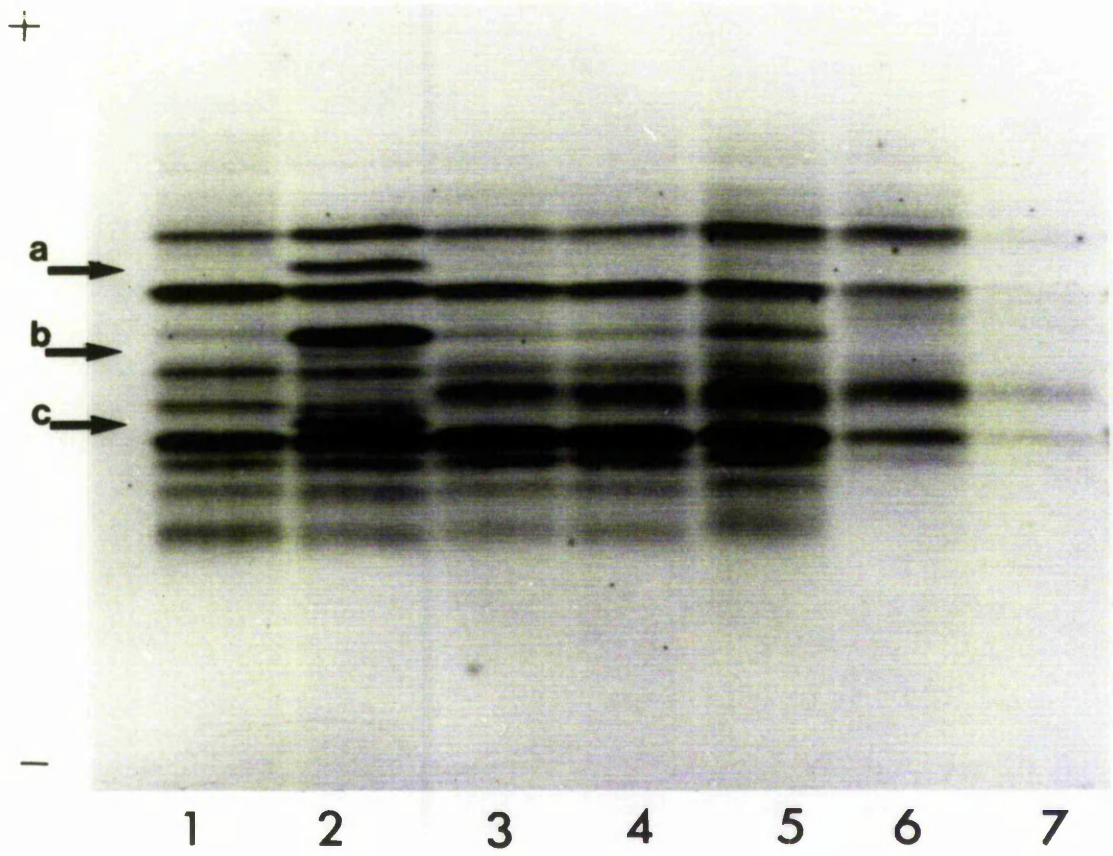

+
a
−
1
2
3
4
5
6
7

Figure 3.20. IEF Separation Patterns of Water Soluble Esterases Extracted from Pasta Samples

The samples illustrated are as follows: lane 1, Durum wheat flour; lane 2, common wheat flour; lanes 3-7, represent pasta samples 1-5 respectively.

IEF was carried out according to the method outlined in Figure 3.16.


+
a
b
c
−
1
2
3
4
5
6
7

(samples 1, 2 and 3, Figure 3.20 lanes 3-5), which agreed with polyphenoloxidase results that common wheat adulteration of these smples had taken place. However the IEF patterns representative of pasta samples 4 and 5 were again poorly resolved making it impossible to establish conclusively if these samples were adulterated (Figure 3.20 lanes 6 and 7). Although it cannot be confirmed that pasta samples 4 and 5 were dried at elevated temperatures, the apparent loss of enzyme activity and/or resolution in the electrophoretic patterns of these samples was probably the result of protein denaturation occuring during drying at high temperatures.

Low-mobility ω-gliadins, (characteristic of common wheat Figure 3.21 lane 6), were present in the gliadin electrophoretic pattern of pasta samples 1 and 2 (Figure 3.21, lanes 5, 4), and absent in similar profiles obtained for pasta samples 3, 4 and 5 (Figure 3.21 lanes 3, 2 and 1). This result again indicated that pasta samples 1-2 were adulterated with common wheat. Similarily on the tlc separation profiles obtained for the petroleum ether extracts of pasta 1 and 2 a strong band was observed at Rm 5.0 which is normally a much weaker band as seen for pasta samples 3-5. This indicated that adulteration by common wheat was present in pasta samples 1 and 2 and absent in pasta samples 3, 4 and 5 (Figure 3.22).

A major peak characteristic of common wheat gliadins (eluting between 47-49 min), was present in the gliadin RP-HPLC profiles of pasta samples 1, 2 and 3 (Figure 3.23). The presence of this peak (marked by an arrow, Figure 3.23) indicated that the samples were adulterated with common

Figure 3.21. Acid PAGE Separation Patterns of Alcohol Soluble Gliadins Extracted from Pasta Samples

The samples illustrated are as follows: lanes 1-5, pasta samples 5-1 respectively; lane 6, common wheat flour; lane 7, Durum wheat flour.

Acid PAGE was carried out according to the method outlined in Figure 3.15.

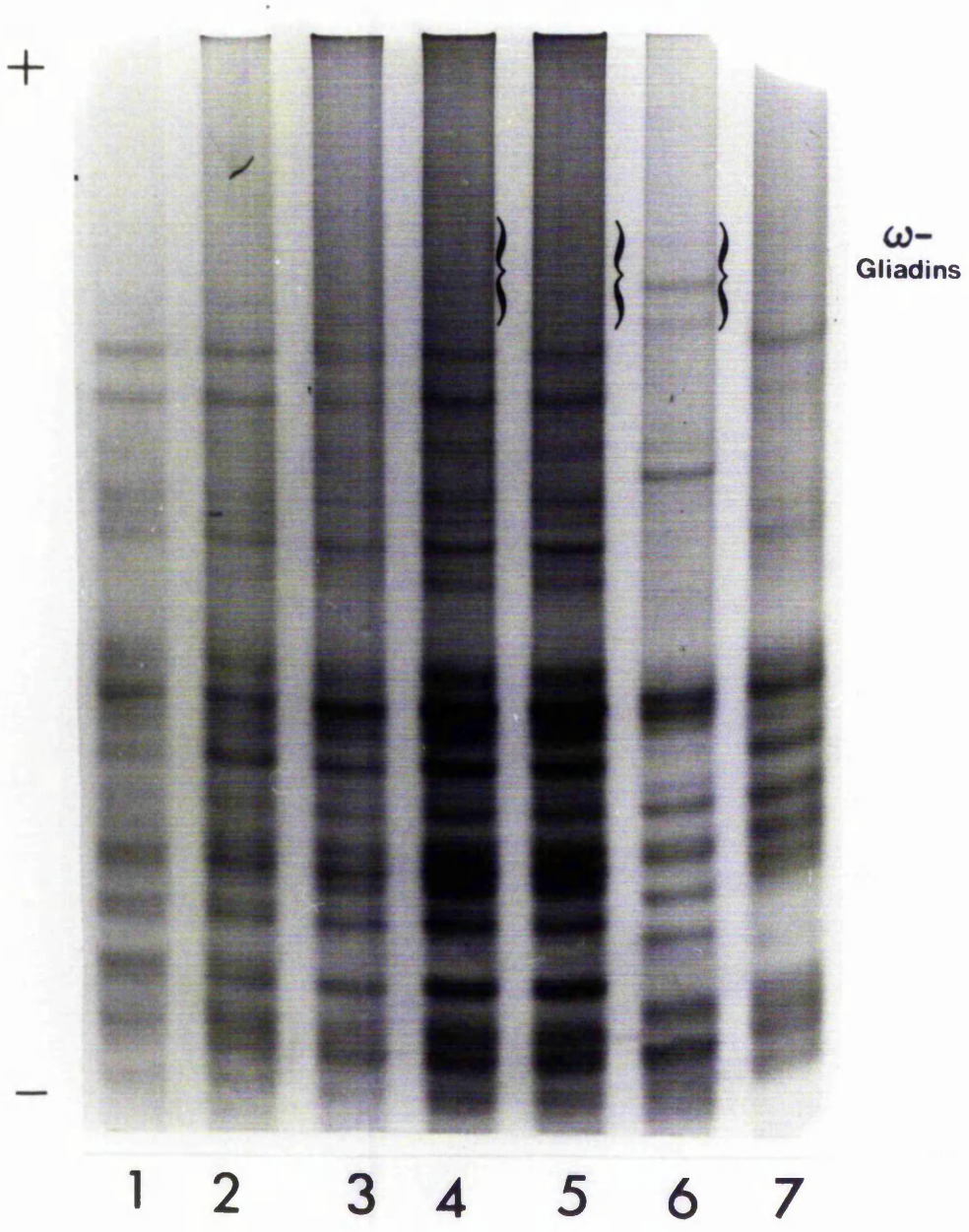

+
−
ω-
Gliadins
1
2
3
4
5
6
7

Figure 3.22. TLC Separation Patterns of Pet-Ether Soluble Fatty Acid Sterols Extracted from Pasta Samples

Lanes 1-5 represent pastas 1-5 respectively. TLC was carried out according to the method outlined in Figure 3.17.

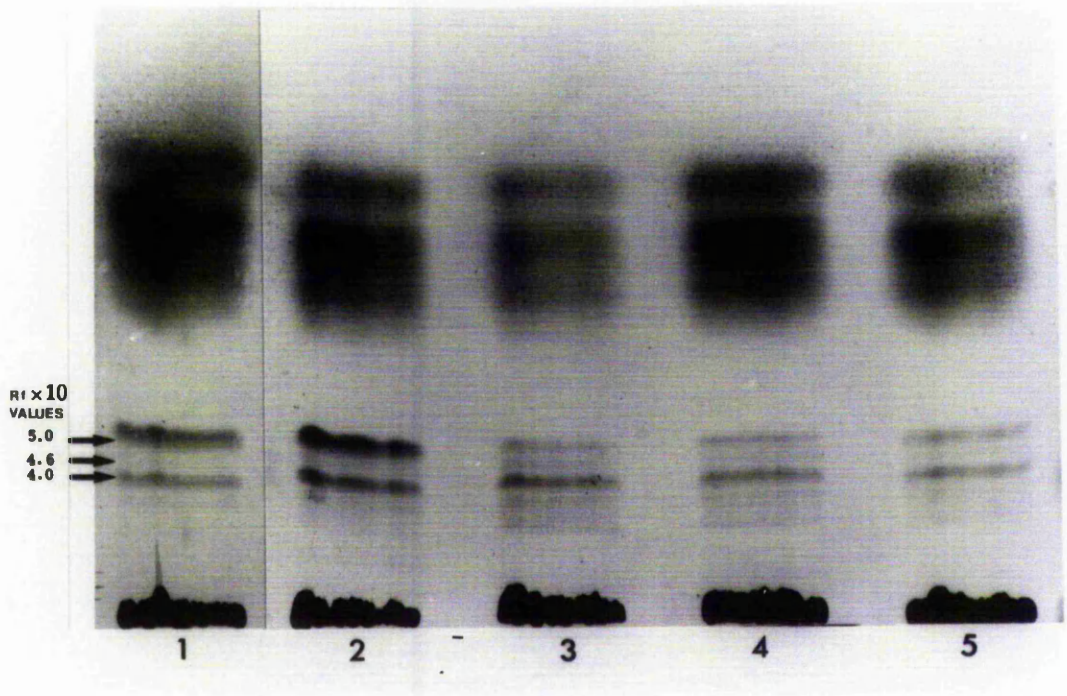
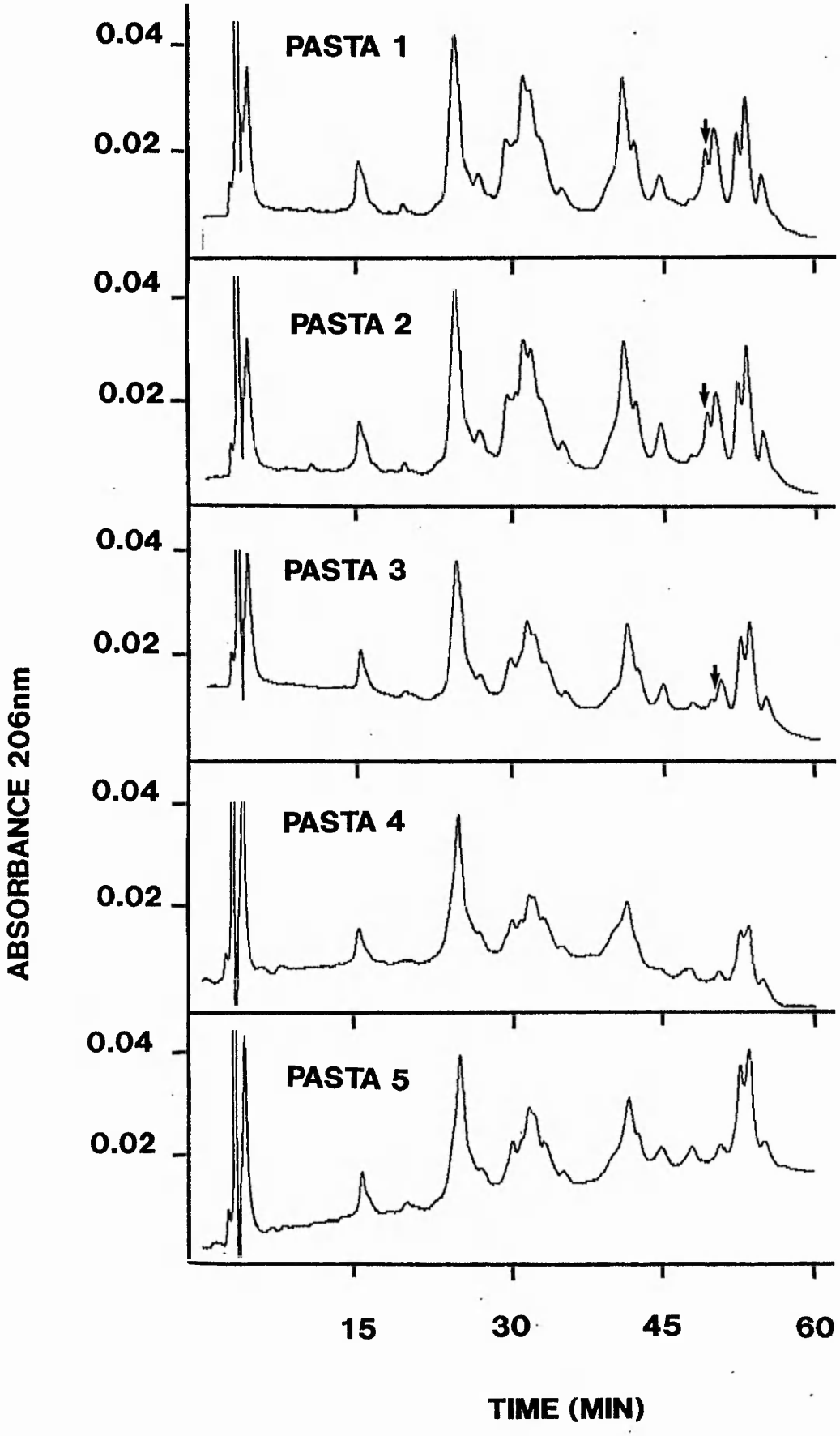

Rf × 10
VALUES
5.0
4.6
4.0
1
2
3
4
5

Figure 3.23. RP-HPLC Separation Profiles of Alcohol Soluble Gliadins Extracted from Pasta Samples

The profiles illustrated represent pastas 1-5 respectively. Extracts (~60μg of protein) were loaded onto a Waters, Delta pak, C18, 5μm particle size column (3.9x 150mm) and eluted according to the method outlined in Figure 3.18.


PASTA 1
PASTA 2
PASTA 3
PASTA 4
PASTA 5
0.04
0.02
ABSORBANCE 206nm
15
30
45
60
TIME (MIN)

wheat. The extent of adulteration, 24 (+/-6%), 22 (+/-6%) and 11 (+/-3%) for samples 1, 2 and 3 respectively, was calculated from a standard curve (Figure 3.24). This curve related the ratio of the RP-HPLC peak (47-49 min) area / protein applied to the column, calculated for pasta manufactured on a laboratory extruder with known levels (0-30%) of common wheat adulteration (as described earlier). Accordingly from this analysis samples 4 and 5 appeared to be manufactured from pure Durum wheat (Figure 3.23).

3.2.2.1. Adulteration Detection Using Electrophoresis following RP-HPLC

As a further means of confirming the RP-HPLC data specific fractions eluting from the RP-HPLC separation of gliadins extracted from pasta 1 and pasta 5 were concentrated by freeze drying. The concentrated protein fractions eluting at intervals between 15-20 min (containing ω-gliadins) and 47-49 min (containing β-/γ-gliadins) were separated by acid PAGE. As shown earlier in Figure 3.21 (lanes 5 and 1 respectively) common wheat specific ω-gliadins were present in the gliadin electrophoretic profile of pasta 1 and absent in pasta 5. These electrophoretic separations are compared in Figure 3.25A (lanes 6, 7 respectively) with the electrophoretic pattern of the gliadins collected between 15-20 min from the RP-HPLC separation of pastas 1 and 5. ω-Gliadins common to both Durum and common wheat eluted between 15-17 min (lanes 3, 4 for pasta 1, 5 respectively). However in pasta 1, common wheat specific ω-gliadins were identified between 17-20 min (Figure 3.25A lane 1). These gliadins were absent in similar fractions collected from

Figure 3.24. Standard Curve Relating Peak Ratio with the Level of Hexaploid Wheat in a Pasta Sample

Gliadins extracted from adulterated (0-30%) pasta manufactured on a laboratory extruder was separated by RP-HPLC according to the method outlined in Figure 3.18.

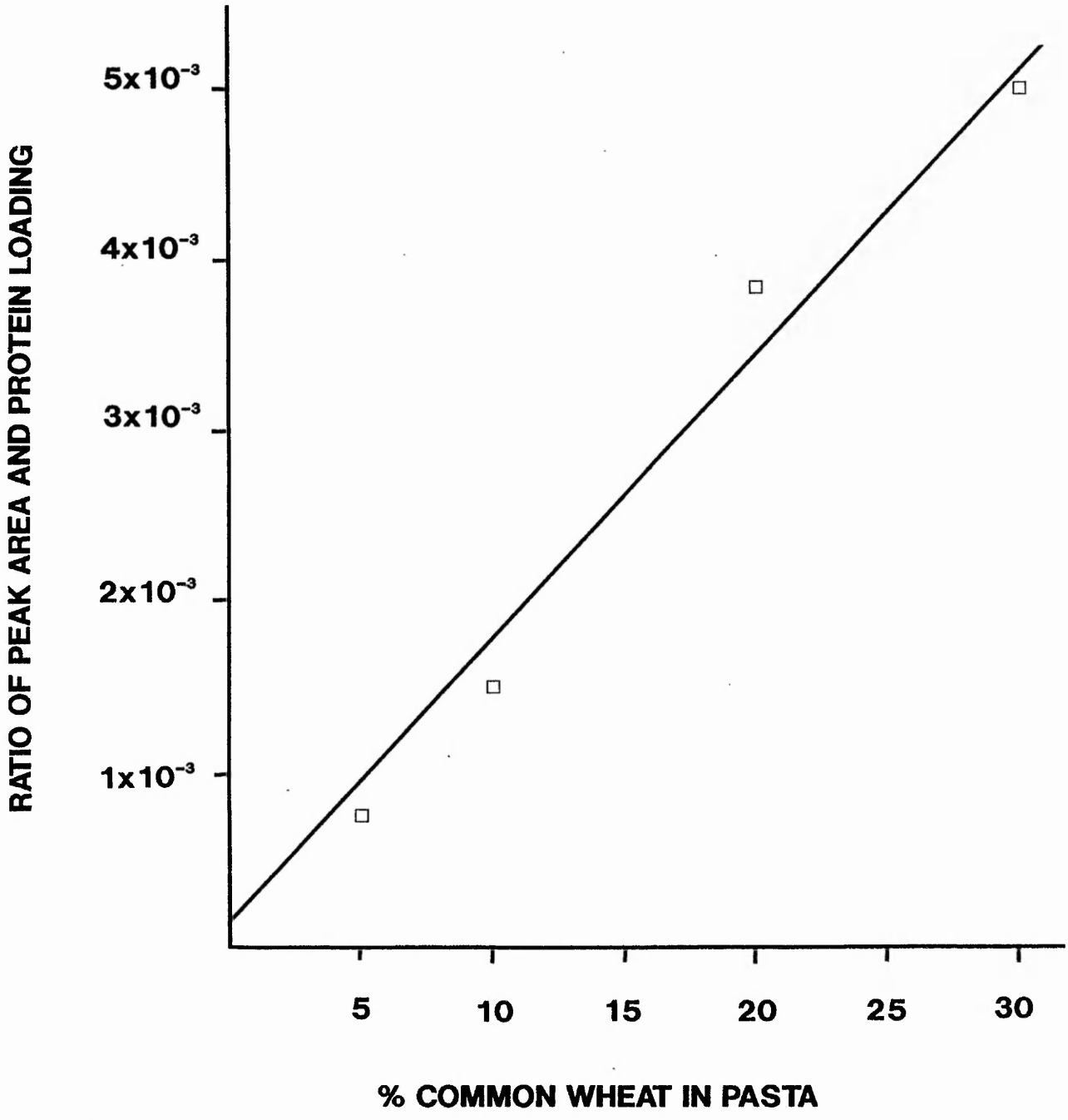

RATIO OF PEAK AREA AND PROTEIN LOADING
5x10⁻³
4x10⁻³
3x10⁻³
2x10⁻³
1x10⁻³
5
10
15
20
25
30
% COMMON WHEAT IN PASTA

Figure 3.25. Electrophoretic Pattern of RP-HPLC Separated Gliadins Extracted of Pasta 1 and Pasta 5

The chromatographic conditions used are outlined in section 2.4.1. and section 2.6.

The fractions illustration are as follows.

A. Lanes 1-2. Fractions collected between 17-20 min for pasta 1 and 5 respectively

Lanes 3-4. Fractions collected between 15-17 min for pasta 1 and 5 respectively

Lane 5. Total Avalon gliadins

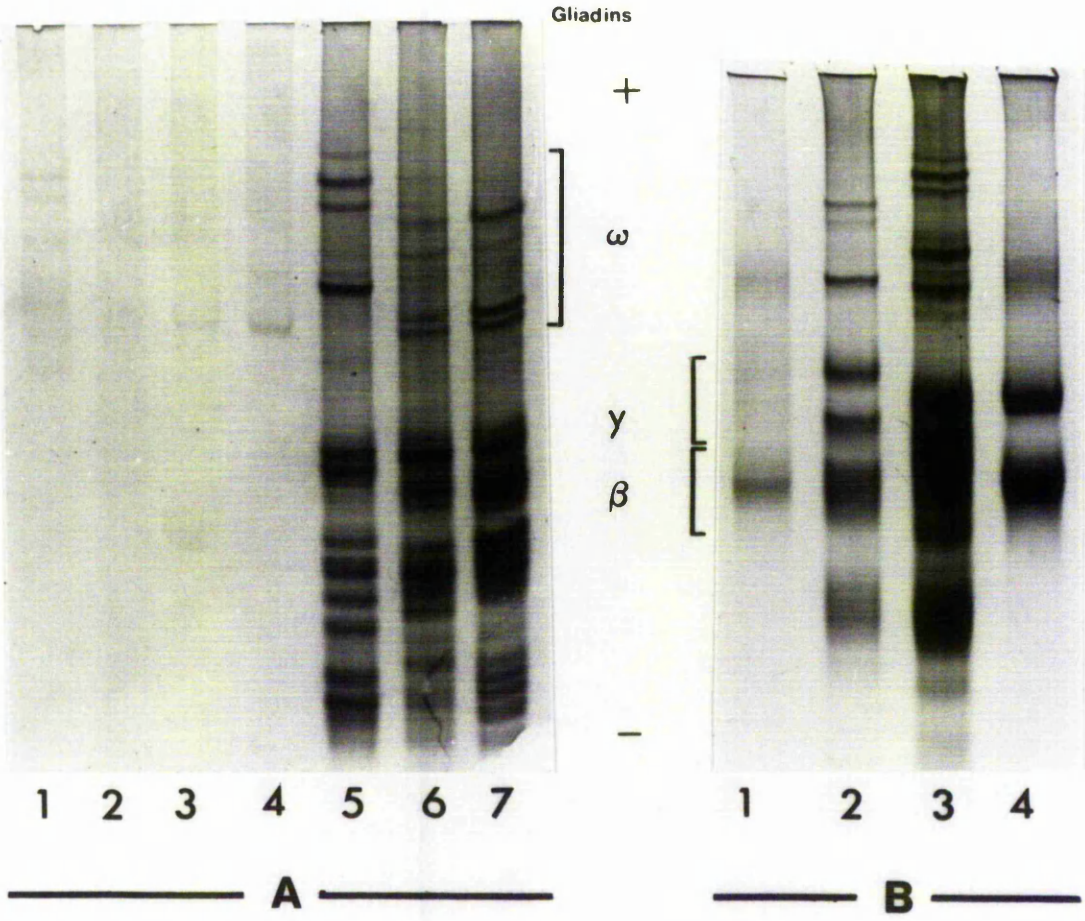
Lane 6-7. Total extract of Pasta 1 and 5 respectively

B. Lane 1. Pasta 5 fraction collected between 47-49 min

Lane 2. Total extract of pasta 5

Lane 3. Total extract of pasta 1

Lane 4. Pasta 1 fraction collected between 47-49 min.


Gliadins
+
ω
γ
β
−
1 2 3 4 5 6 7
A
1 2 3 4
B

pasta 5 (Figure 3.25A lane 2). In the fractions collected between 47 and 49 minutes in pasta sample 5 only one band of β-gliadin was found, (Figure 3.25B, lane 1). This was normally the case with Durum wheat (section 3.1.3.2). In contrast in pasta sample 1 (Figure 3.25B, lane 4) β/γ-gliadin bands were found which were usually only present in common wheat (section 3.1.3.2). This combination of RP-HPLC and acid PAGE facilitates sensitive detection of common wheat specific gliadins in an adulterated pasta sample and provides confirmation of a result from either technique.

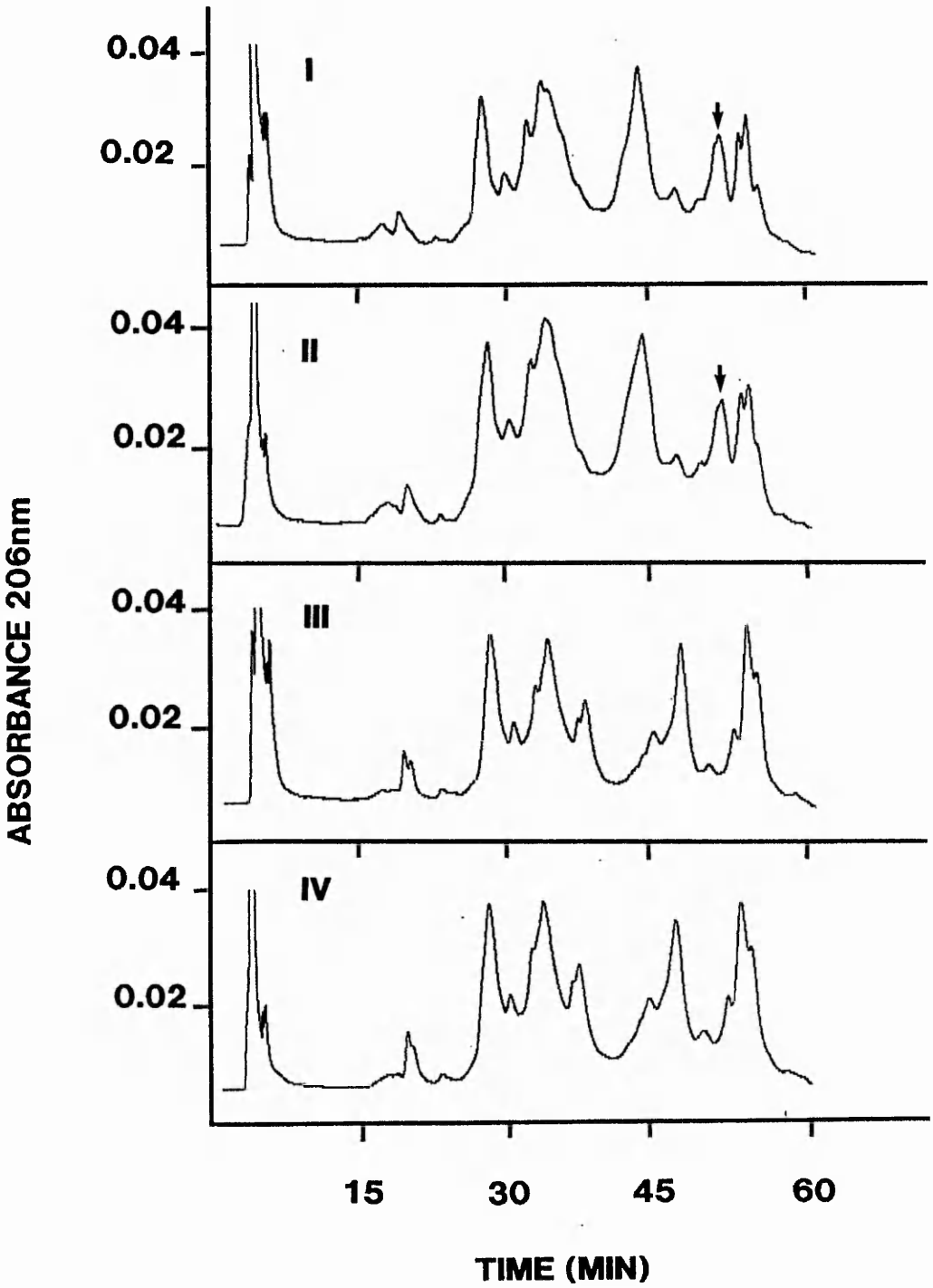
3.2.2.2. Reversed-Phase HPLC of Gliadins from Adulterated Pasta Samples Dried at High Temperatures

In order to authenticate the RP-HPLC method it was necessary to test it on adulterated pasta samples dried at high temperatures.

Pasta samples 1 (containing traces of common wheat) and pasta sample 2 (manufactured from 100% Durum wheat) were rehydrated and redried at 85^{o}C for 4h. The gliadin RP-HPLC profiles of these pastas chromatographed before and after drying were similar (Figure 3.26), with little or no evidence of protein denaturation. This showed that gliadin RP-HPLC profiles were suitable for detecting common wheat adulteration in pasta dried at elevated temperatures and conformed with earlier data (Figure 3.23) carried out on unadulterated pasta samples. Extracts of the same pasta taken before and after the redrying step showed a complete loss of resolution and/or enzyme activity in alkaline PAGE and IEF electrophoregrams of grain polyphenoloxidases and esterases respectively.

Figure 3.26. RP-HPLC Profiles of Gliadin Extracted from Pasta Samples 1 and 5 before and after Drying at 85°C.

Profiles i and ii represent pasta 1 before and after heating and profiles iii and iv represent pasta 5 before and after heating respectively. Gliadin extracts (60μg of protein) were loaded onto a Waters, Delta pak, C18, 5μm particle size column (3.9x 150mm) and eluted according to the method outlined in Figure 3.18.


0.04
0.02
I
II
III
IV
ABSORBANCE 206nm
15
30
45
60
TIME (MIN)

3.2.3. Discussion

Heat denaturation of wheat gluten as measured by a loss of protein functionality was reported by Schofield et al. (1983) to occur mainly between 55°C and 75°C, with 90% of the denaturation ocurring within the first 2 min at that temperature. Similar results showing a time / temperature relationship for the denaturation of wheat protein have been reported by other authors, who concluded that gluten and especially water soluble wheat proteins, after exposure to high temperatures, were very susceptible to heat damage (Wu and Iglett, 1974; Pence et al., 1953). Consequently analytical methods designed to assess protein composition must not be sensitive to changes caused by high temperatures (unless the effects of temperature are specifically under study).

During extrusion, pasta is exposed to temperatures between 45-50°C during a residence time of less than 1 min. This includes the time required for the dough mixture to reach the desired temperature. It is unlikely that denaturation of wheat protein occurs under these conditions. However "normal" drying conditions (40°C for 22h) are now replaced by "superheat" drying cycles involving temperatures (85°C for 4h) sufficient to denature gliadin and water soluble wheat proteins. Because of this it is of the utmost importance that assays used for the detection of common wheat adulteration in pasta products are unaffected by high temperature processing conditions. Of the methods examined in this report this appeared to be the case for methods based on the tlc separation of phytosterol palmitate and acid PAGE

separation of gliadin proteins. Neither appeared to be severely affected by heating at elevated temperatures, however interpretation of results by these two methods is highly subjective with any quantitative measurements being at best an estimate.

From this study it is clear that with the possible exception of acid PAGE of gliadins, the electrophoretic methods currently used for detection of common wheat adulteration of pasta products are susceptible to a loss of sensitivity when applied to pasta dried at high temperatures. This effect is most clearly seen in the alkaline PAGE and IEF patterns of polyphenoloxidases and esterases respectively, where the loss of enzymatic activity and resolution make it extremly difficult to detect the presence or absence of common wheat adulteration in pastas dried at high temperature. Alkaline PAGE of specific polyphenoloxidases extracted from pasta is the method recommended in France and Italy for detection of common wheat adulteration of pasta. Argueably this method gives an accurate indication of the adulteration status of pasta dried at normal temperatures but it is shown in this study to be unsuitable for analysis of pasta dried at high temperature.

It has also been shown that detection methods based on the analysis of water soluble wheat proteins are unsuitable for the analysis of pasta dried at high temperatures. It was also considered that methods which rely on tlc separation of phytosterol palmitate or the electrophoretic separation of gliadin protein were unsuitable because of the subjective nature of the results.

A method was therefore sought which could be used to detect common wheat adulteration of Durum pasta and provide results which could be easily interpreted and quantified if necessary. RP-HPLC separation of gliadin proteins appeared to satisfy these criteria. The method was shown to be equally applicable to the detection of common wheat adulteration in low temperature and high temperature dried pasta and in the original semolina flour. Although protein denaturation in high temperature pasta was shown to cause some peak distortion in the RP-HPLC profile, the area of interest, i.e. the late eluting gliadins were unaffected by the drying temperatures used in this study. Visual inspection of the elution profile was used to detect common wheat adulteration in a series of pasta samples while quantitation of adulteration levels was made possible by extrapolation of results to a standard curve constructed for pasta containing levels of adulteration between 0-30% (m/m) common wheat.

These investigations indicate that any technique which is used routinely for detecting common wheat adulteration of pasta products must satisfy the following biochemical and operational criteria: the technique must be based on measurement of a component of common wheat which is independant of environmental and processing conditions; it must be possible to identify and quantify the component in an efficient, accurate and reproducible manner; optionally for laboratories with a high sample throughput the technique should be amenable to automation. The only technique which complies with these criteria is that based on the RP-HPLC

separation of gliadin proteins. This can reproducibly detect low levels (down to 5%) of common wheat adulteration in pastas within 90 min of receipt of sample.

When laboratories require additional more definitive information e.g. in cases of dispute, a more complex procedure is described. This involves RP-HPLC of the sample gliadins, collection and concentration of common wheat specific proteins from the analytical column and further examination of these characteristic proteins by acid PAGE. Thus information gathered from two systems one chromatographic the other electrophoretic used in series may be used to provide confirmational evidence.

3.3. CHARACTERISATION OF HEXAPLOID WHEAT SPECIFIC GLIADINS

The variation in the genomic composition of hexaploid and tetraploid wheat results in wheat varieties having different gliadin electrophoretic patterns (Metakovsky et al., 1984). The most prominent differences are observed in the ω-gliadin region of the gel where slow eluting ω-gliadins are found only in hexaploid wheat varieties (Cooke et al., 1986). γ-Gliadins have also been found which are coded by the D-genome and are consequently found in hexaploid wheat and absent in Durum wheat (Shewry et al., 1983).

By electrophoretic analysis of fractions collected from the RP-HPLC separation of gliadins it is known that late eluting ω-gliadins found in the electrophoretic pattern of Avalon and absent in D. Valdur elute between 17-18 min while early eluting ω-gliadins common to both species elute between 15-17 min (section 3.1.3.2). Similarily for Avalon the major peaks eluting between 47 and 49 min have been shown, also using electrophoresis to contain eight bands of γ/β-gliadin protein (section 3.1.3.2). Only one gliadin band, responsible for a small peak, elutes at this time on the gliadin RP-HPLC profile of Durum Valdur.

These differences, detected using a combination of gliadin RP-HPLC and/or electrophoresis have been used as a basis for techniques to detect common wheat adulteration of Durum wheat and pasta products.

Since the biochemical characteristics of ω-gliadins are already well documented (Charbonnier, 1974) only the Rm values pertaining to the electrophoretic

separation of these proteins will be reported here. The biochemical characteristics of hexaploid wheat specific γ-/β-gliadins eluting between 47-49 min on the RP-HPLC separation of total gliadin extracts of Avalon are compared with electrophoretically separated γ-/β-gliadins obtained from a total gliadin extract of Avalon, Slejpner and Durum Valdur gliadins. Furthermore these gliadins are discussed in relation to similar proteins separated by Ewart (1977); Bietz et al. (1977) and Popineau and Pineau (1985).

3.3.1. Determination of Electrophoretic Rm Values

Rm values of ω-, γ- and β-gliadin proteins were calculated from the one dimensional electrophoretic patterns of the wheat varieties D. Valdur, Avalon and Slejpner according to the method of Sapirstein and Bushuk (1985) as described in section 2.4.1.5. using a total extract of Neepawa as the standard cultivar.

i) ω-Gliadins

The wheat varieties D. Valdur, Avalon and Slejpner each had 4, 7 and 10 bands of ω-gliadins respectively (Figure 3.27). These varieties were chosen as representative examples of Durum and hexaploid wheat varieties. However natural variation in the genomic composition of different wheat varieties results in the existence of other ω-gliadin bands which are not mentioned in this study. Avalon and Slejpner each had the following ω-gliadins as determined by one dimensionl electrophoresis, 13, 16, 18, 25, 27, 34 and 35. In addition bands 29, 31 and 32 were found in Slejpner. ω-Gliadin bands 20, 23, 29 and 31 were found in the electrophoretic pattern of D. Valdur none of which were found in Avalon but ω-gliadin 31 was also found in the variety Slejpner.

ii) γ-/β-Gliadins

Using the gliadin classification system described above the electrophoretic mobility of the γ- and β-gliadin bands in the wheat varieties Avalon, Slejpner and D. Valdur was determined (Figure 3.27). Thus, a total extract of Avalon had γ-gliadin bands 42, 44, 46, 47, 48, 49 and β-gliadin bands 50, 53, 54, 56, 57, 61, 63, 64. A total extract

Figure 3.27 Determination of Gliadin Electrophoretic Mobility.

Total gliadins and gliadins recovered from fractions collected following the RP-HPLC separation of a total gliadin extracts of Avalon are separated using the chromatographic conditions outlined in section 2.4.1. and section 2.6.

The fractions illustrated are as follows:

Lane 1. Total Avalon gliadins

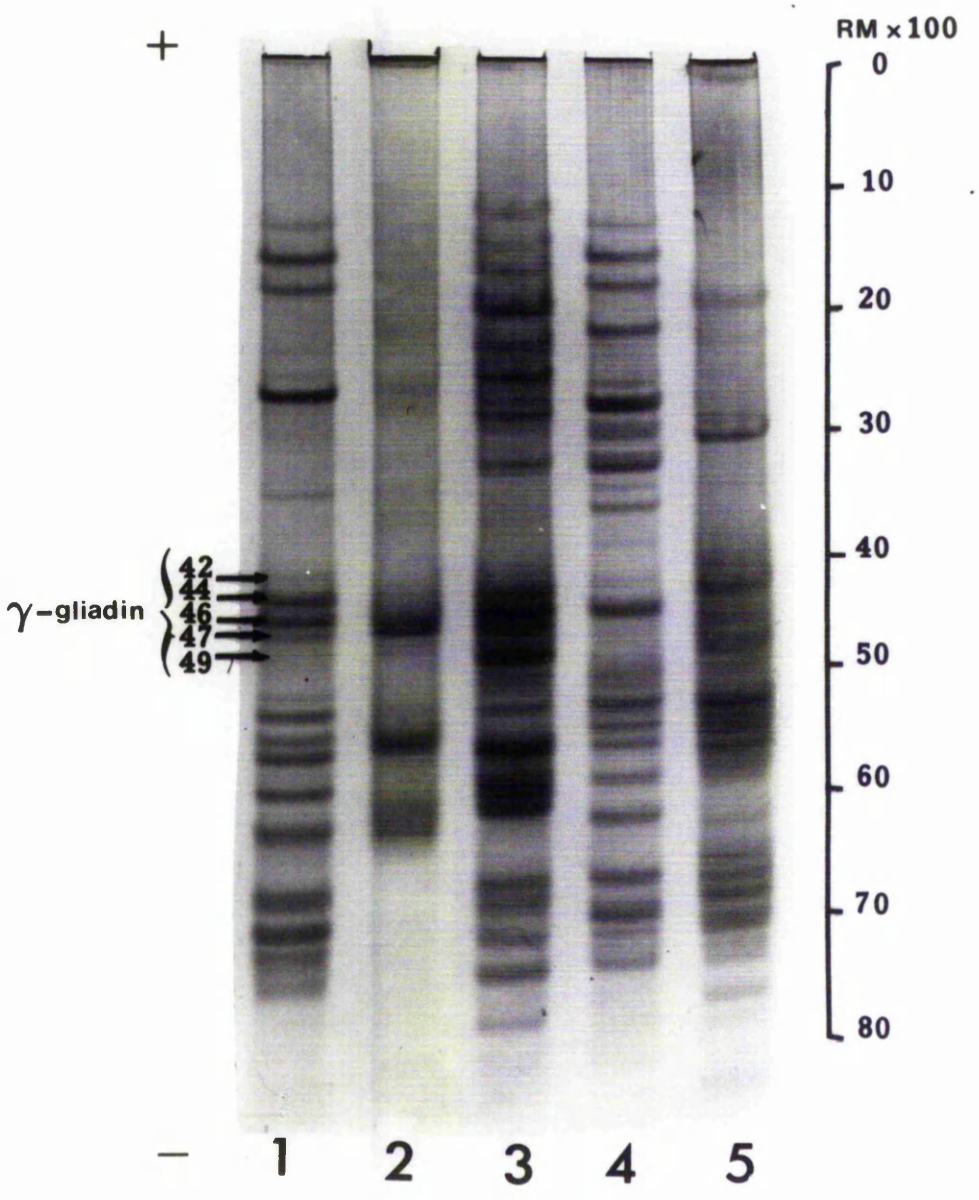
Lane 2. Avalon fraction collected between 47-49 min

Lane 3. Total Neepawa gliadins

Lane 4. Total Slejpner gliadins

Lane 5. Total D. Valdur gliadins

Rm values were calculated according to the method of Sapirstein and Bushuk (1985).


+
RM x100
0
10
20
30
40
50
60
70
80
γ-gliadin
42
44
46
47
49
–
1
2
3
4
5

of Slejpner had γ-gliadin bands 42, 44, 47 and β-gliadin bands 50, 52, 53, 54, 56, 57, 61, 63. A total extract of D. Valdur had γ-gliadin bands 42, 45, 45.5, 48, and β-gliadin bands 50, 52, 55, 55.5, 56.5, 58, 61, 64.

iii) RP-HPLC Fractionated Gliadins

Avalon gliadin protein fractionated by RP-HPLC between 47-49 min was identified electrophoretically as γ-gliadin 44, 46, 47, and β-gliadin 54, 56, 57, 63, 64. The latter two bands were also found in the RP-HPLC fraction eluting between 50-55 min and were thus not considered specific to this RP-HPLC fraction. γ-Gliadin 46 and β-gliadin 56 appeared more densely stained in this RP-HPLC fraction suggesting that these proteins were predominantly present. Other hexaploid wheat gliadins (Moulin, Maris Huntsman, Hobbit) separated under similar conditions had 6 gliadins (γ-44, 46, 47 and β-54, 56, 57) eluting between 47 and 49 min, in these varieties, gliadin bands with electrophoretic mobilities of 63 and 64 were absent.

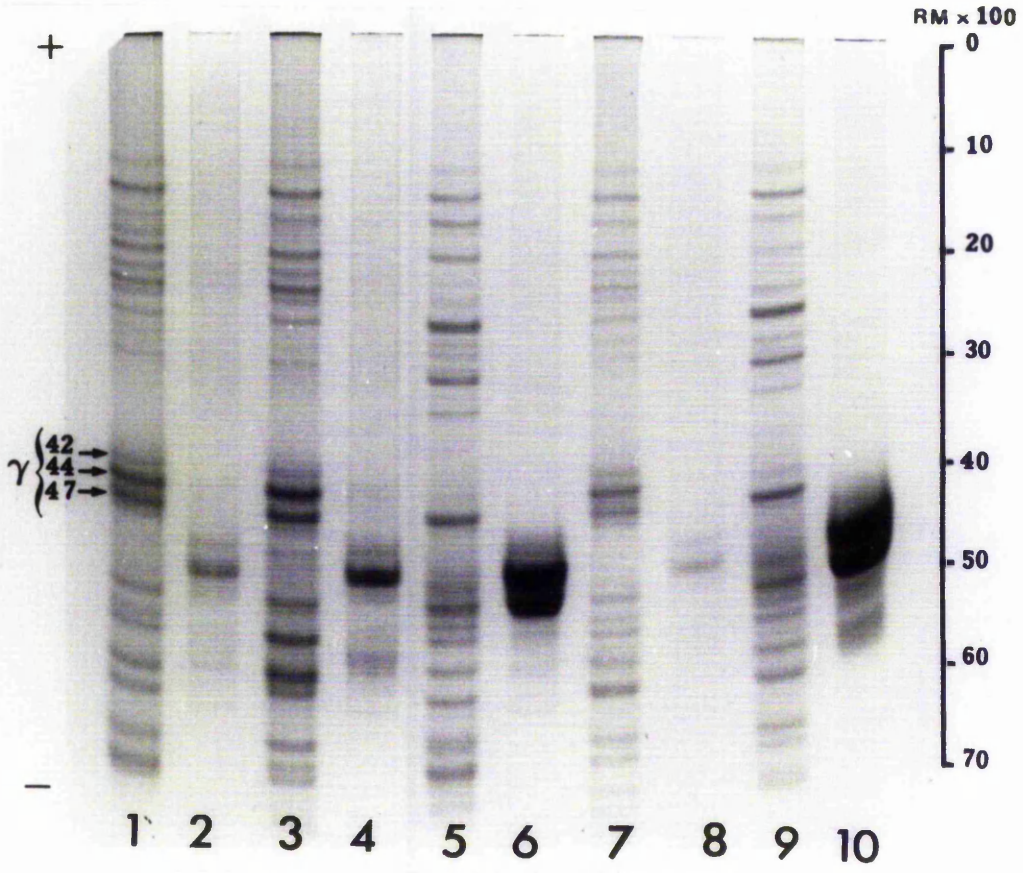
Five hexaploid wheat varieties (Longbow, Riband, Slejpner, Norman, Apollo) were found which had only 5 gliadin components eluting in the RP-HPLC fraction collected between 47-49 min (Figure 3.28). These were electrophoretically identified as β-gliadins 50, 52, 56, 61, 63 (Figure 3.28-note that due to a "edging effect" on the gel lane 10 has not fully resolved). No γ-gliadins eluted in these fractions although γ-gliadins, with mobilities corresponding to 42, 44 and 47 were seen on the total gliadin electrophoretic pattern obtained for each variety.

The single band of protein eluting between 47 and

Figure 3.28. Gliadins Electrophoretic Pattern of Unusual Hexaploid Wheat Varieties

Total extracts from the Wheat Varieties Longbow, Riband, Slejpner, Norman and Apollo are shown in lanes 1, 3, 5, 7, 9 respectively and the electrophoretic pattern of the protein fractions collected between 47-49 min during RP-HPLC separation of these varieties is shown in lanes 2, 4, 6, 8, 10 respectively. The chromatographic conditions used are outlined in section 2.4.1. and section 2.6.

Rm values were calculated as outlined in Figure 3.27.


+
−
γ
42
44
47
RM x 100
0
10
20
30
40
50
60
70
1
2
3
4
5
6
7
8
9
10

49 min on the gliadin profile of D. Valdur was identified as β-gliadin 64 (Figure 3.10, section 3.1).

The γ-gliadin composition of the above wheats are summarised as follows:

	Gliadin							
	γ-42	γ-44	γ-45	γ-45.5	γ-46	γ-47	γ-48	Y-49
Avalon	+[a]	+++	-[b]	-	+++	+	+	+
Slejpner	+	+++	-	-	-	+	-	-
D. Valdur	+	-	+	++	+	-	+	-
RP-HPLC Fraction	-	+	-	-	+++	+	-	-

[a]-Indicates a band is present, the intensity of staining is represented by more than one plus sign. [b]-Indicates the band is absent.

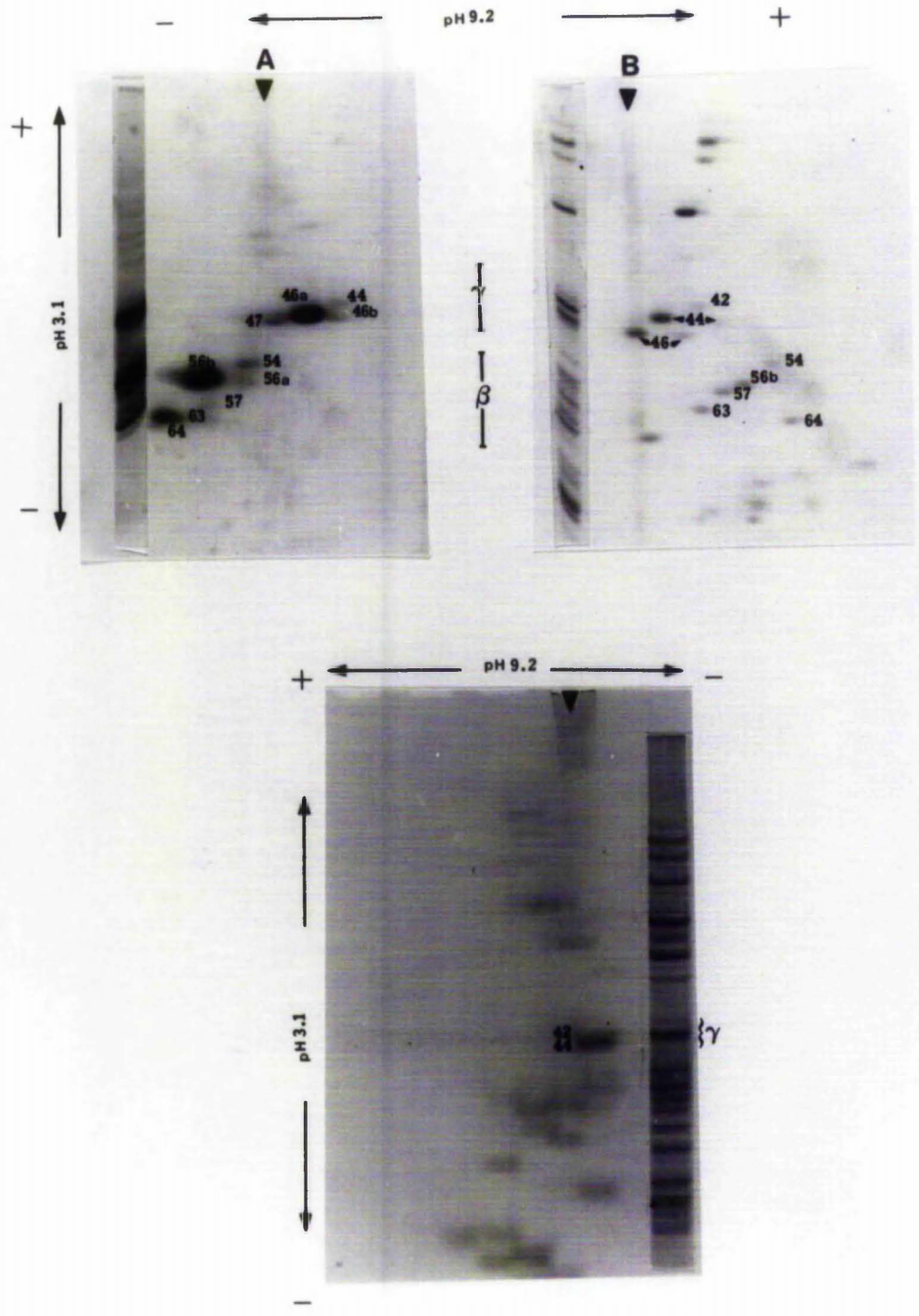
3.3.2. Characterisation of β- γ-Gliadins

3.3.2.1. Two Dimensional PAGE of Gliadins at Two pH Values

Two dimensional PAGE separating gliadins at pH 3.1 in the first dimension and at pH 9.2 in the second dimension was carried out on Avalon γ/β gliadins fractionated by RP-HPLC (γ-44, 46, 47, and β-54, 56, 57, 63, 64) (Figure 3.29A) and total gliadin extracts of Avalon and Slejpner (Figure 3.29B; 3.29C). It was shown in section 3.3.1. that the γ- and β-gliadin composition of the hexaploid wheat varieties Apollo, Slejpner, Longbow, Riband and Norman differed from that of Avalon. The variety Slejpner was then chosen as representative of these 5 varieties and its γ-/β-gliadin composition was investigated. γ-Gliadins did not elute between 47-49 min during RP-HPLC of gliadins extracted from

Figure 3.29. Two-dimensional Electrophoretic Separation of Gliadins Extracted from Hexaploid Wheat

The patterns illustrated are (A) RP-HPLC fractionated γ/β-gliadins (B) total Avalon gliadins extracted in 70% v/v aq ethanol and (C) total Slejpner gliadins extracted in 70% v/v aq ethanol. The first dimension separation (vertical axis) was carried out at pH 3.2 and the second dimension separation (horizontal axis) was carried out at pH 9.2 as outlined in section 2.4.4. ▼ indicates origin.


pH 9.2
A
B
pH 3.1
46a
44
47
46b
56b
54
56a
57
63
64
γ
β
42
44
46
54
56b
57
63
64
pH 9.2
pH 3.1
γ

these 5 varieties. Thus this RP-HPLC fraction was not separated by 2 dimensional electrophoresis. Using this technique it was hoped to differentiate between the different γ/β-gliadins observed in the first dimension and check their homogeniety in relation to their mobility at pH 9.2.

i) RP-HPLC Separated Gliadins (Figure 3.29A)

In the second dimension, after migrating towards the anode RP-HPLC fractionated γ-44 and 47 obtained from Avalon each gave one major band with γ-44 migrating further than γ-47. γ-46 separated into two bands consisting of one major (46a) and one minor band (46b) both migrating anodically. γ-46b (migrated further than 46a) had similar mobility to γ-44. β-Gliadins 54, 56, 57, 63, and 64 all migrated cathodically. This shows that even at pH 9.2 these components bear a positive charge, which may be a result of their very low content of ionized glutamic and aspartic acid residues (Popineau and Pineau, 1985). β-Gliadins 54, 57, 63, and 64 each gave essentially one major band while β-56 gave two bands, one large (56b) and one small (56a).

ii) Total Gliadin Extracts of Avalon and Slejpner

The 2D PAGE pattern for the RP-HPLC fractions were compared with those obtained for total gliadin extracts of Avalon and Slejpner. In the Avalon 2D-gliadin pattern (Figure 3.29B) γ-42 (which did not elute between 47-49 min on gliadin RP-HPLC) appeared as a single anodic band. γ-44 consisted of two anodic bands, one major and one minor band. γ-46 also consisted of two bands one major (with no apparent mobility) and one minor anodic band. Bands corresponding to γ-47, β-54 and β-56a were no longer be detected. β-Gliadins

57, 63, 64 which previously migrated cathodically now all migrated anodically.

In the second dimension, after migrating anodically γ-42 from a total extract of Slejpner appeared as one band, γ-44 also appeared as one major band of protein but with no mobility under alkaline PAGE conditions. γ-47 was no longer detected. β-Gliadins 50, 52, 53, 54, 56, 57, 61, 63 each gave essentially one major band and migrated anodically.

3.3.2.2. Isoelectric Focussing of Gliadin Proteins

Isoelectric focussing (IEF) of total gliadin extracts of Avalon and D. Valdur revealed the presence of many protein bands in the pH range 5-10 (Figure 3.30). IEF of Avalon γ-/β-gliadins fractionated by RP-HPLC between 47-49 min revealed that four different gliadin bands were present in this fraction. These gliadins having isoelectric points (pI) of 6.5, 6.8, 7.0, were also resolved on the isoelectric profiles of total extracts of Avalon and Slejpner but absent on the IEF profile of D. Valdur. A gliadin band with a pI = 9.6 was found only on the gliadin IEF profile of Avalon and absent on D. Valdur and Slejpner. Isoelectric points were calculated by comparison with the following standards: phycocyanin (pI = 4.60); β-lactoglobulin B (pI = 5.10); bovine carbonic anhydrase (pI = 6.00); human carbonic anhydrase (Pi=6.50); equine myoglobin (pI = 7.00); lentil lectin (pI = 8.40); cytochrome C (pI = 9.60).

3.3.2.3. Molecular Weight Determination of Gliadins

Avalon γ/β gliadins fractionated by RP-HPLC (γ-44, 46, 47, and β-54, 56, 57, 63, 64) (Figure 3.29A) and total

Figure 3.30 Isoelectric Focussing Patterns of Gliadins

Isoelectric Focussing (between pH 3-10) was carried out as outlined in section 2.4.5.2. The profiles illustrated are as follows:

Lane 1. IEF standards

Lane 2. Total Durum gliadins

Lane 3. Total Slejpner gliadins

Lane 4. Total Avalon gliadins

Lane 5. IEF standards

Lane 6. γ/β-gliadins

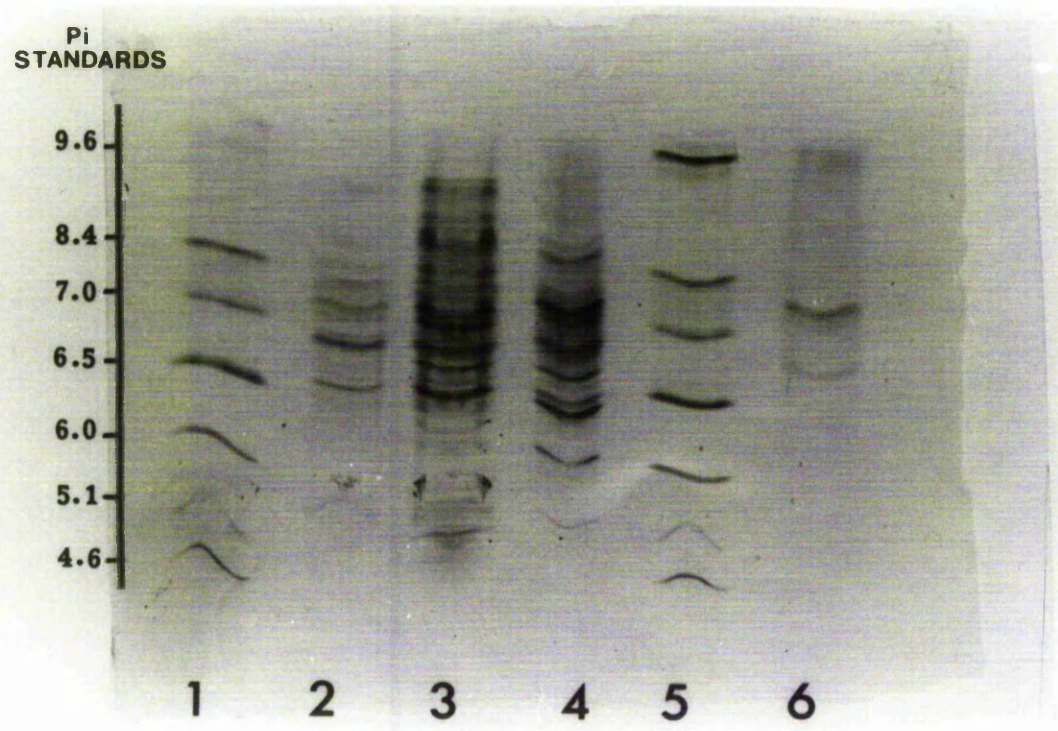
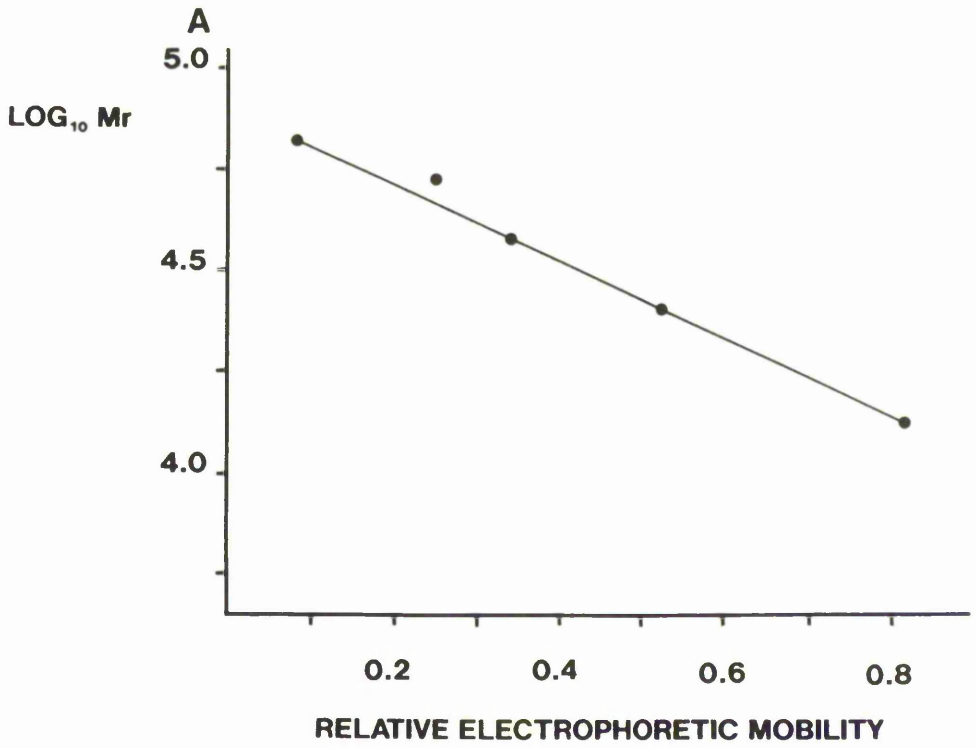

Pi
STANDARDS
9.6
8.4
7.0
6.5
6.0
5.1
4.6
1
2
3
4
5
6

Figure 3.31 Gliadin Molecular Weight Determination using Gradient SDS-PAGE.

A. Calibration curve relating the molecular weight markers (carbonic anhydrase (30,000), egg albumin (45,000), bovine serum albumin (67,000), phosphonylase B (94,000) and β-galactosidase (116,000)) and their respective Rm values.

B. SDS-PAGE of total Avalon gliadins (lane 1), total D. Valdur gliadins (lane 2) RP-HPLC fractionated γ/β-gliadins (lane 4), and molecular weight markers (lane 3).


A
5.0
LOG10 Mr
4.5
4.0
0.2
0.4
0.6
0.8
RELATIVE ELECTROPHORETIC MOBILITY


B
Mr x 10-3
116
94
67
45
30
γ-gliadin
β-gliadin
1
2
3
4
−
+

gliadin extracts of Avalon and D. valdur were subjected to gradient SDS-PAGE (10-20%) after reduction of disulphide bonds with 2-mercaptoethanol. Molecular weights were estimated from a standard curve (Figure 3.31A) drawn from the electrophoretic mobilities of the following standards: carbonic anhydrase (30,000), egg albumin (45,000), bovine serum albumin (67,000), phosphonylase B (94,000) and β-galactosidase (116,000). Figure 3.31B shows the SDS PAGE pattern for total Avalon and D. Valdur gliadins and the RP-HPLC fractionated gliadins. The molecular weight of γ-gliadin 44, 46 and 47 was estimated to be 45,000, that of β-gliadin 54, 56 and 57 was 35,000 and that of β-gliadins 63 and 64 was 20,000.

3.3.3. Discussion

The RP-HPLC fractionation of crude Avalon gliadins did not produce pure gliadin components as evidenced by the heterogeneity of the protein eluting between 47-49 min. However its use as a prelimimary fractionation procedure seems promising. The number of distinct gliadin components purified between 47-49 min appeared to depend upon whether deamidation of glutamine residues occurred during RP-HPLC fractionation. If deamidation occurred, 10 distinct proteins (γ-44, 46a, 46b, 47 and β-54, 56(a), 56(b), 57, 63, 64,) were obtained as detected by 2D acid PAGE. If deamidation did not occur during fractionation then only 9 proteins were obtained. In this case the minor component of β-56 (56a) would merely be a deaminated form.

γ-Gliadin 44 of Avalon was shown to be composed of two different proteins, 44 major with slow mobility and 44

minor with faster mobility at pH 9.2. Only the minor band was separated in the fraction collected between 47-49 min during RP-HPLC. This indicated that the hydrophobicity of the two γ-44 gliadins was significantly different. The same effect was seen in Figure 3.10 (section 3.1) where γ-44 minor and γ-44 major were detected in two different chromatographic peaks which eluted using different acetonitrile concentrations. γ-Gliadin 46 separated by RP-HPLC between 47-49 min was composed of two proteins similar to the γ-46 obtained from a total gliadin extract of Avalon. But the electrophoretic mobility at pH 9.2 of γ-46 major separated by RP-HPLC was faster than that of γ-46 from a total extract.

β-Gliadin 56 isolated by RP-HPLC gave two bands, (56b and 56a) while the β-56 obtained from the total extract consisted of only one protein. Similar discrepencies between gliadins prepared in acidic and alcoholic solutions were claimed by Charbonnier (1974) and Popineau and Pineau (1985). These workers explained the differences as artefactual heterogeneity, occuring as a result of deamidation of aspartamine and glutamine residues. As RP-HPLC separation of gliadin proteins was carried out at pH 2.5 and samples were stored in this buffer for about 24h prior to freeze drying it is possible that β-56a is a deamidated version of β-56b. This partially explained the heterogenity of β-56 when the total extract and the RP-HPLC separated fraction were compared. It must however be emphasized that heterogeneity may exist in these gliadin which is not observed on the 2D pattern of a total gliadin extract of Avalon because of too little protein being electrophoresed. This was possibly what happened in

the case of bands corresponding to γ-47, β-54 and β-56b which cannot be detected on the 2D pattern for total Avalon gliadins.

Artefactual heterogeneity does not adequately explain the shift in migration pattern observed for β-gliadin 57, 63, 64 which as a result of RP-HPLC separation appeared to shift from anodic to cathodic mobility. This effect was possibly as a result of conformational differences occuring when the RP-HPLC fractionated proteins, which unfolds during RP-HPLC separation, refolds with a different conformation at the end of RP-HPLC. The refolded molecule looses its surface hydrophobicity when it refolds in an aqueous solution and is likely to be replaced by more hydrophillic amino acid residues. β-Gliadin has a high concentration of basic amino acids, especially arginine (Table 1.4) and a lower content of acidic amino acids which contribute to give the protein a nett positive charge even at pH 9.2. The deamidated gliadin β-56a, however has a neutral charge brought about by the negative charge of deamidation being balanced by the native positive. The anodic mobility of γ-Gliadins is reflected by the higher content of acidic amino acids and lower content of basic amino acids (Table 1.4) found in these proteins. This helps to explain the differences seen between the 2D separation patterns of the RP-HPLC fractionated gliadins and that of the total extract of gliadins.

Three γ-gliadins (γ1, γ2, γ3,) were purified by Huebner et al. (1967), in order of their elution from a sulphoethyl cellulose column. Two of these were later purified independently and termed γ-1st peak and γ-2nd peak

(Ewart, 1977). Ewart (1977) showed that γ-1st peak is equivalent to γ2 and γ-2nd peak was equivalent to γ3. These gliadins all had identical mobilities on starch gel electrophoresis at acid pH. The data shown in this section is in agreement with that of Popineau and Pineau (1985) indicating that each of these γ-gliadins had a different mobility in acid PAGE. Popineau and Pineau (1985) proposed that γ-44 is of the γ3 type (molecular weight > 40,000 and a low tyrosine content) and γ-46 is of the γ2 type (molecular weight <40,000, low tyrosine content). These authors also showed that γ-gliadin fractions differed from each other in terms of their methionine and glutamine contents, their degree of amidation and their surface hydrophobicity.

The data presented in this section confirmed the electrophoretic heterogeneity of the Y-gliadins 44 and 46. The different RP-HPLC retention times shown in this section and in section 3.1 further proved that the two proteins have different hydrophobicities. The 2D electrophoretic pattern of β-56 also confirmed that deamidation of this protein was occuring as a result of low pH fractionation and that in vivo β-56 exists as one band of protein which lacks mobility at pH 9.2. No deamidation of γ-46 was detected as a result of low pH storage as previously reported by Popineau and Pineau (1985).

On the basis of their biochemical characteristics and the absence of γ-44, 46a, 46b, 47 in Durum wheat varieties a study was conducted to investigate the immunochemical characteristics of these proteins.

3.4 DETECTION OF COMMON WHEAT ADULTERATION OF DURUM WHEAT AND PASTA BY IMMUNOCHEMICAL DETECTION OF γ-GLIADINS

To date all immunochemical methods proposed to detect and quantify common wheat adulteration of Durum wheat and pasta products use antisera raised to wheat cytoplasmic proteins (Piazzi and Cantagelli, 1969; Piazzi et al., 1972; Pompucci et al., 1978; Cantigelli et al., 1979). The susceptibility of cytoplasmic proteins to changes resulting from environmental growth conditions (Wrigley and Shepherd, 1973) and to denaturation as a result of the high temperatures used during pasta manufacture (Wu and Iglett, 1974; Pence et al., 1953) cast doubt on the reliability of these methods.

Immunochemical analysis of wheat storage proteins has been limited by their low solubility in aqueous buffers and by their low antigeneity (Vu and Popineau, 1987) and also by the problems of non-specific reactions particularily in ELISA systems (Ayob et al., 1988). In addition it is difficult to obtain pure gliadin components for the preparation of specific antibodies (Vu and Popineau, 1987). However some successful studies of wheat storage proteins have revealed the presence of immunologically distinct glutenins (Escribano and Graber, 1966; Escribano, 1967) and gliadins (Elton and Ewart, 1963; Ewart, 1966; Nimmo and O'Sullivan, 1967) but extensive antibody cross reactivity between all the various prolamins has been demonstrated (Festenstein et al., 1987). Earlier reports (Beckwith and Heiner, 1966) suggested that the antigenic determinants of

both α and β-gliadins were missing on γ-gliadins and Vu and Popineau (1987) subsequently showed that two γ-gliadins labelled 44 and 46 carry common antigenic determinants. These authors demonstrated that antisera raised against γ-44 and γ-46 did not cross-react with other types of gliadins (α-, β- or ω) and it was concluded that antigenic determinants of γ-gliadin components correspond to polypeptide structures that do not exist in other gliadins.

In this study, polyclonal antibodies were raised to two gliadin fractions (a) a total extract of Avalon gliadins and (b) RP-HPLC fractionated γ/β gliadins eluting between 47-49 min. After adsorbing out anti-Durum wheat gliadin IgG, using Durum gliadins bound to sepharose 4B, the cross reactivity of the bound and unbound IgG with hexaploid and Durum wheat varieties was assessed using the immunoblotting technique. In this way an immunochemical based test was developed which could be used for the detection of common wheat adulteration of Durum wheat and pasta products.

3.4.1 Modification of ELISA For Detection of Gliadins

The immune response of rabbits to successive doses of gliadins was monitored using a non-competitive heterologous enzyme linked immunosorbent assay (ELISA). This assay involves immobilising the antigen onto plastic wells and then binding rabbit anti-gliadin antibody in serum to the antigen. The use of ELISA for gliadin detection and quantitation has been reported by Keyser and Mahler (1973); Ciclitira and Lennox (1983); Windemann et al. (1982); Ciclitira et al. (1985); Gosling et al. (1985); McKillop et al. (1985); Skerritt (1985); Troncone et al. (1986). However inconsistent gliadin binding patterns makes many of these tests unreliable (Ayob et al., 1988). Therefore before applying the standard ELISA procedure to the analysis of gliadins a numbers of different binding protocols were investigated to help establish a reproducible system.

Gliadins (2.5 mg/ml) in 70% ethanol were diluted to 0.25, 0.50, 0.75, 1.00, 2.00 μg/50μl with each of the following solutions: 2M urea; 70% v/v aq ethanol; PBS. Diluted antigen (50μl) was applied to selected wells of an ELISA plate. Gliadins diluted in 2M urea and PBS were allowed to bind as in the standard ELISA procedure. Gliadins in 70% (v/v) ethanol were bound by allowing the ethanol to evaporate. A fourth assay was utilised whereby the PBS diluted gliadin sample was applied to an ELISA plate pre-treated with poly-L-lysine and glutaraldehyde (section 2.9.2, henceforth this treatment will be referred to as poly-L-lysine treatment). After binding and blocking, the ELISA was performed as outlined in section 2.9.3. Immunoglobulin G

(IgG) the major specific antibody class in immune serum was labelled by binding an anti-rabbit IgG enzyme conjugate (horse radish peroxidase) to the immobilised complex bound to the ELISA well. IgG was then indirectly detected by a simple spectrophotometric enzyme assay (section 2.9.3).

From titration curves (Figure 3.32A) of diluted anti-gliadin antisera, the serum dilution at 50% antigen saturation was calculated for each antigen concentration (0.25-2.00 μg/well) in the different buffer systems (values calculated for 2μg gliadin/well were omitted in Figure 3.32A as these only differed from values obtained for 1 μg/well when gliadins were treated with poly-L-lysine). These values were used to calculate a relationship between antigen concentration and 50% antigen saturation (Figure 3.32B). From Figure 3.32B (excluding the response of gliadins bound by allowing ethanol to evaporate from the ELISA wells) it can be seen that at concentrations of antigen greater than 0.30 μg/well significant variability was observed in the antibody titer calculated for each antigen concentration, (although the antibody concentration was similar for all). Thus it was decided that different amounts of gliadin were binding to each well even though the maximum binding capacity of an ELISA well is known to be 1μg of protein (Becton Dickinson Labware). Pre-treatment of the ELISA wells with poly-L-lysine increased the maximum binding of antigen, and indicated a direct correlation between the concentration of antigen bound and the antibody titer. Dilution of antigen with 2M urea and with PBS also lead to the detection of high antibody titers for anti-gliadin antisera (25,000, 20,000

Figure 3.32. Titration Curves of Test Sera Obtained from Rabbits Immunised with Total Avalon Gliadins

Varying levels (0.25μg ●, 0.50μg □, 0.75 μg ▲, 1.0μg ◆) of gliadins were bound to the untreated Micro-Titer ELISA Plates in different buffers systems as follows:

A i Gliadins solubilised in 2M urea

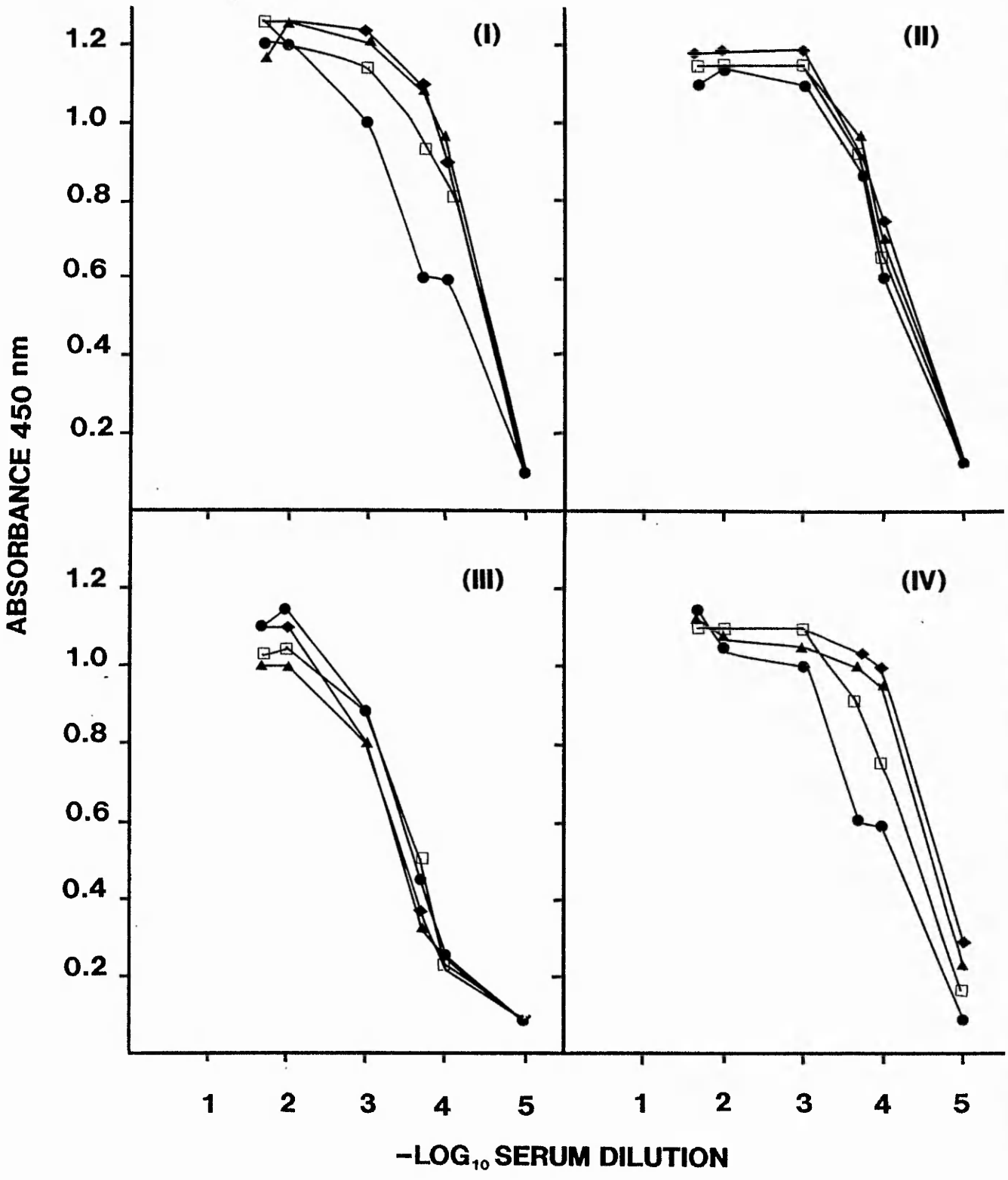
ii 70% v/v aq ethanol soluble gliadins diluted in PBS

iii Gliadins soluble in 70% v/v aq ethanol applied directly and bound by evaporation of ethanol

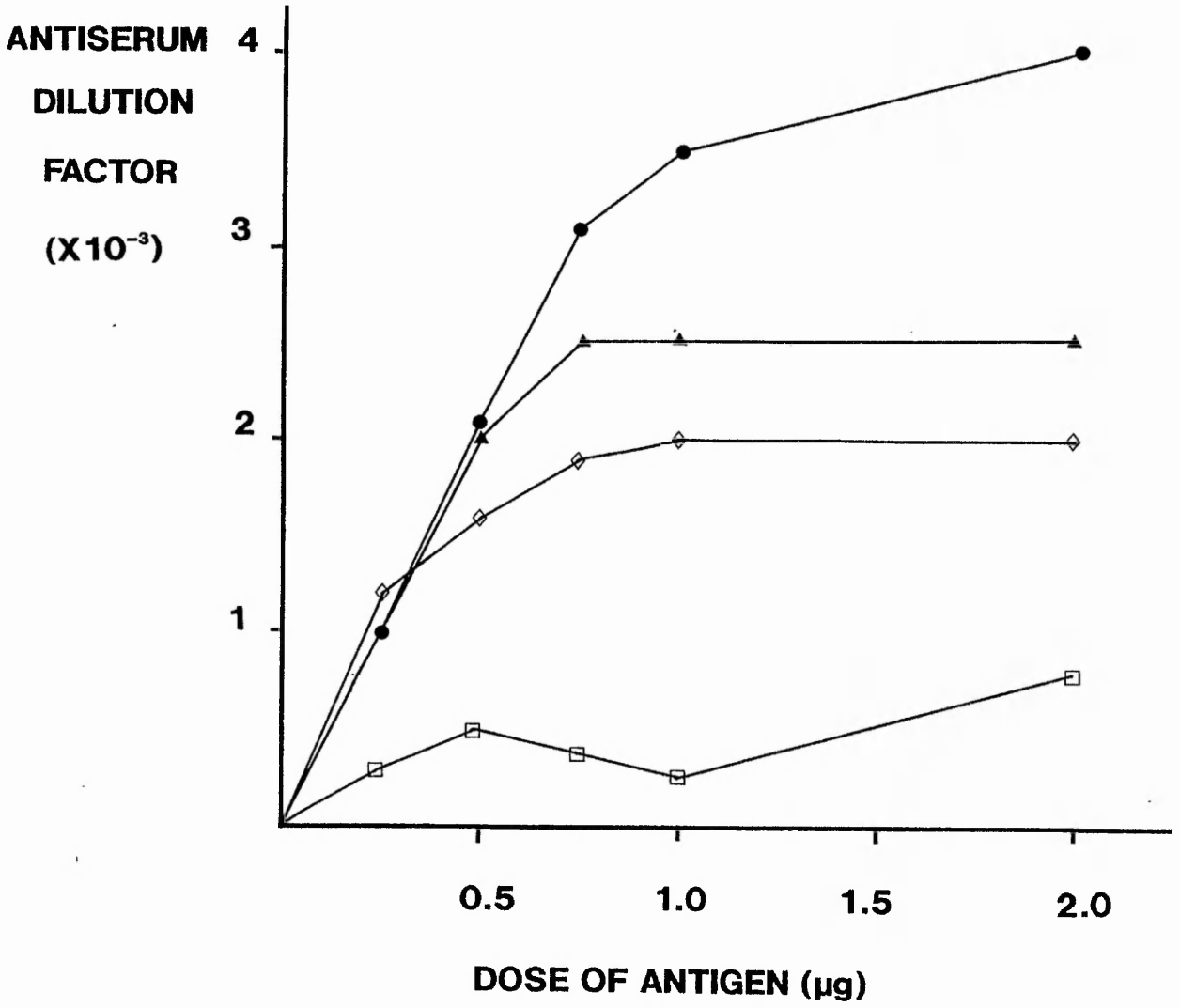
iv 70% v/v aq ethanol soluble gliadins diluted in PBS applied to Micro titer ELISA plates pre-treated with poly-L-lysine and glutaraldehyde.

B Assessment of the immune response as measured by non-competitive ELISA with gliadins bound using the buffer systems listed i-iv above (i= ▲, ii= ◇, iii= □ , iv= ●). Serum dilution factor (reciprocal of serum dilution producing 50% saturation of antigen) was plotted against dose of antigen applied to the ELISA well

A


(I)
(II)
(III)
(IV)
ABSORBANCE 450 nm
1.2
1.0
0.8
0.6
0.4
0.2
1
2
3
4
5
−LOG10 SERUM DILUTION

B


ANTISERUM
DILUTION
FACTOR
(X10⁻³)
4
3
2
1
0.5
1.0
1.5
2.0
DOSE OF ANTIGEN (µg)

respectively) on non poly-L-lysine treated wells but the response was much less than that obtained using PBS diluted antisera on poly-L-lysine treated wells (35,000). Gliadins bound by allowing ethanol to evaporate from the ELISA wells gave a poor response indicating that only a minimum amount of gliadins were binding. From these results it was decided that the binding of gliadins to ELISA wells was best performed with the aid of poly-L-lysine treatment. Therefore all further ELISAs were carried out using this modification.

3.4.1.1. Immune Response to Gliadins

The immune response of rabbits to successive gliadin doses was assessed by measuring the dilution of antiserum required to produce 50% saturation of antigen bound to ELISA wells (an example for total Avalon gliadin is shown in Figure 3.33A). Absorbance values for "No-antigen" controls were subtracted from the test data. Antisera obtained after administering successive antigen doses required successively higher dilutions to produce 50% saturation of bound antigen. Plots of dilution factor against dose of antigen administered, produced a similar immune response pattern for total and RP-HPLC fractionated gliadins (Figure 3.33B). A sudden decrease in immune response was observed after the third immunisation. It was proposed by Hudson and Hay (1980) that this may be as a result of rabbits becoming tolerant to the soluble antigen.

3.4.1.2. Precipitation of IgG from Antisera

Crude anti-gliadin IgG fractions were obtained by ammonium sulphate precipitation of antisera (section 2.10.1) raised against total Avalon gliadins and RP-HPLC fractionated

Figure 3.33. Titration of Rabbit Anti-gliadin Serum by Non-Competitive ELISA

A. Titration curves of test sera obtained from rabbits immunised with total Avalon gliadins using a non-competitive ELISA. Serum dilution was estimated at 50% saturation of antigen (indicated by broken lines from left to right corresponding to successive doses of antigen) bound to a

Micro-Titer ELISA Plate pre-treated with poly-L-lysine and glutaraldehyde (50% maximum absorbance).

Mean values (n=3) are plotted.

B Assessment of the immune response as measured by non-competitive ELISA . Serum dilution factor (reciprocal of serum dilution producing 50% saturation of antigen) was plotted against dose of antigen administered 3 weeks prior to assay.

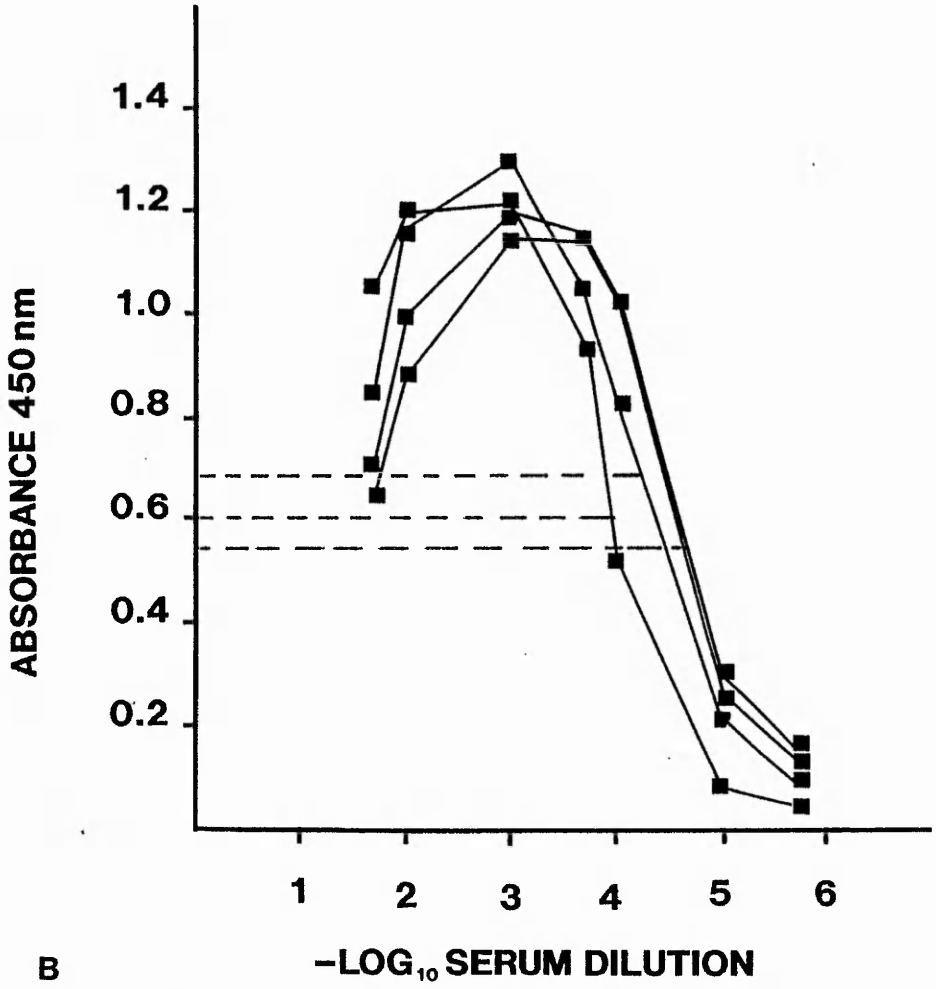
Immune responses are plotted for rabbits immunised with the following antigens:

A. Total Avalon gliadins

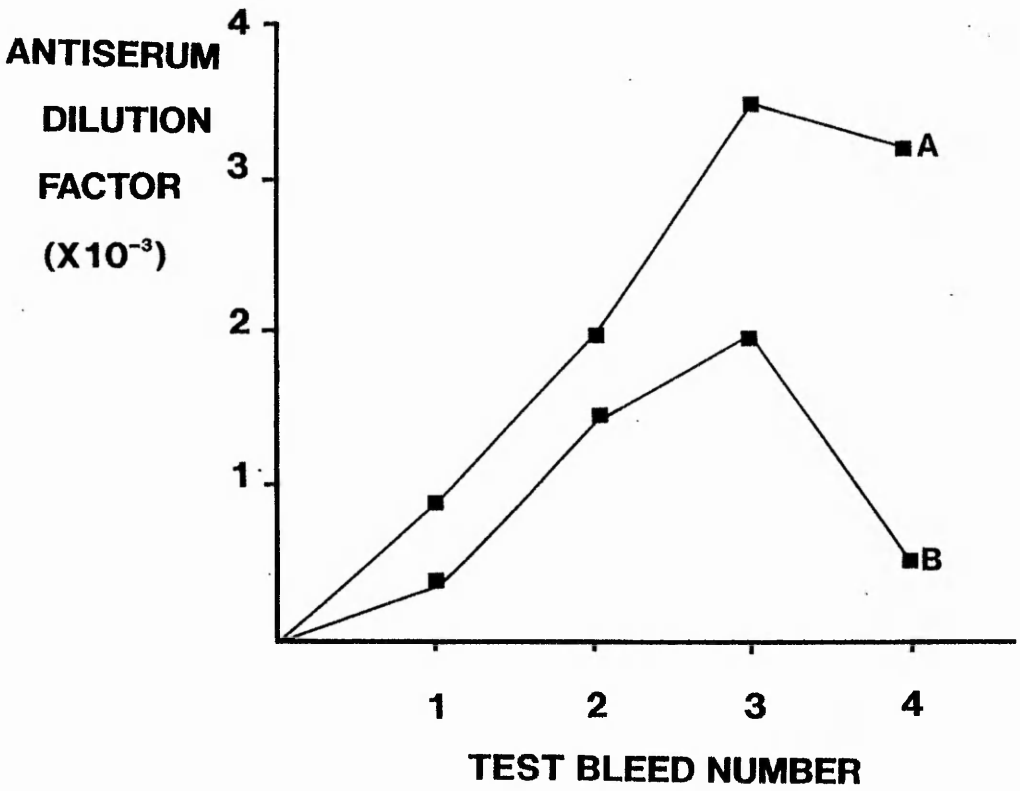
B. RP-HPLC fractionated γ/β-gliadins

Mean values (n=3) are plotted.

A


ABSORBANCE 450 nm
1.4
1.2
1.0
0.8
0.6
0.4
0.2
1
2
3
4
5
6
−LOG10 SERUM DILUTION

B


ANTISERUM
DILUTION
FACTOR
(X10⁻³)
4
3
2
1
A
B
1
2
3
4
TEST BLEED NUMBER

γ/β gliadins. The relative antibody activities were estimated as a function of the dilution of each fraction required to produce 50% saturation of antigen bound to the ELISA well. Absorbance values for "No Antigen" controls were subtracted from the test data. Purification factors of about 1.5 (Table 3.7) were obtained.

3.4.1.3. Cross-reactivity of Anti-gliadin Antisera

The interspecies cross reactivity of anti-gliadin IgG was determined by incubating western blots of hexaploid and Durum wheat gliadins with the antibody preparations and developing the immunoblots using the avidin-biotin reaction to amplify the response. Anti-Avalon gliadin IgG was found to cross-react strongly with α-, β-, γ-, ω-gliadins of Avalon and Durum wheats but reacted weakly with the γ-/β-gliadins fractionated by RP-HPLC (Figure 3.34, lanes 1-3). Anti-gliadin IgG raised to RP-HPLC fractionated γ/β gliadins cross reacted strongly with α-,β-, γ-, ω-gliadins of hexaploid wheat (both total and RP-HPLC fractions) and with ω-/γ-gliadins of Durum wheat (Figure 3.34, lanes 4-6).

Since anti-gliadin IgG raised to RP-HPLC fractionated γ/β-gliadins cross reacted with Durum wheat gliadins, it was not used, in its complete form to detect common wheat adulteration of Durum wheat. Previous data (section 3.1.3.2) had indicated that common wheat specific γ-gliadin (as determined by RP-HPLC and electrophoresis) was present in this fraction which was absent in Durum wheat. For this reason it was decided to use a total gliadin extract of D. Valdur to adsorb out anti-Durum gliadin IgG molecules present in the anti-gliadin IgG raised to hexaploid wheat.

Table 3.7 Isolation of IgG from Rabbit Serum

FRACTION	VOL (ml)	RELATIVE ANTIBODY ACTIVITY (units/ml)	TOTAL RELATIVE ANTIBODY ACTIVITY (units)	TOTAL PROTEIN (mg)	SPECIFIC ACTIVITY (units/mg)	RECOVERY %	PURIFICATION
Serum A[1]	25	64	1600	463	3.5		-
Ammonium Sulphate Dialysate	12.5	112.5	1406	269	5.2	88	1.5
Serum B[2]	25	10	250	375	0.6	-	-
Ammonium Sulphate Dialysate	12.5	20	250	213	1.2	100	1.9

1 unit = antibody dilution factor ($X10^{-4}$) producing 50% saturation of antigen bound to the ELISA plates under ELISA conditions. Ammonium sulphate precipation was carried out as outlined in sectio 2.10.1.

1: Antisera raised in rabbits immunised with a total extract of gliadins from the cultivar Avalon

2: Antisera raised in rabbits immunised with γ-/β-gliadins fractionated between 47-49 min during RP-HPLC separation of gliadins extracted from the cultivar Avalon

Figure 3.34 Immunoblots Produced after Incubation of Western Blots of Total Gliadin Extracts with Anti-gliadin Anti-sera.

The samples illustrated are as follows:

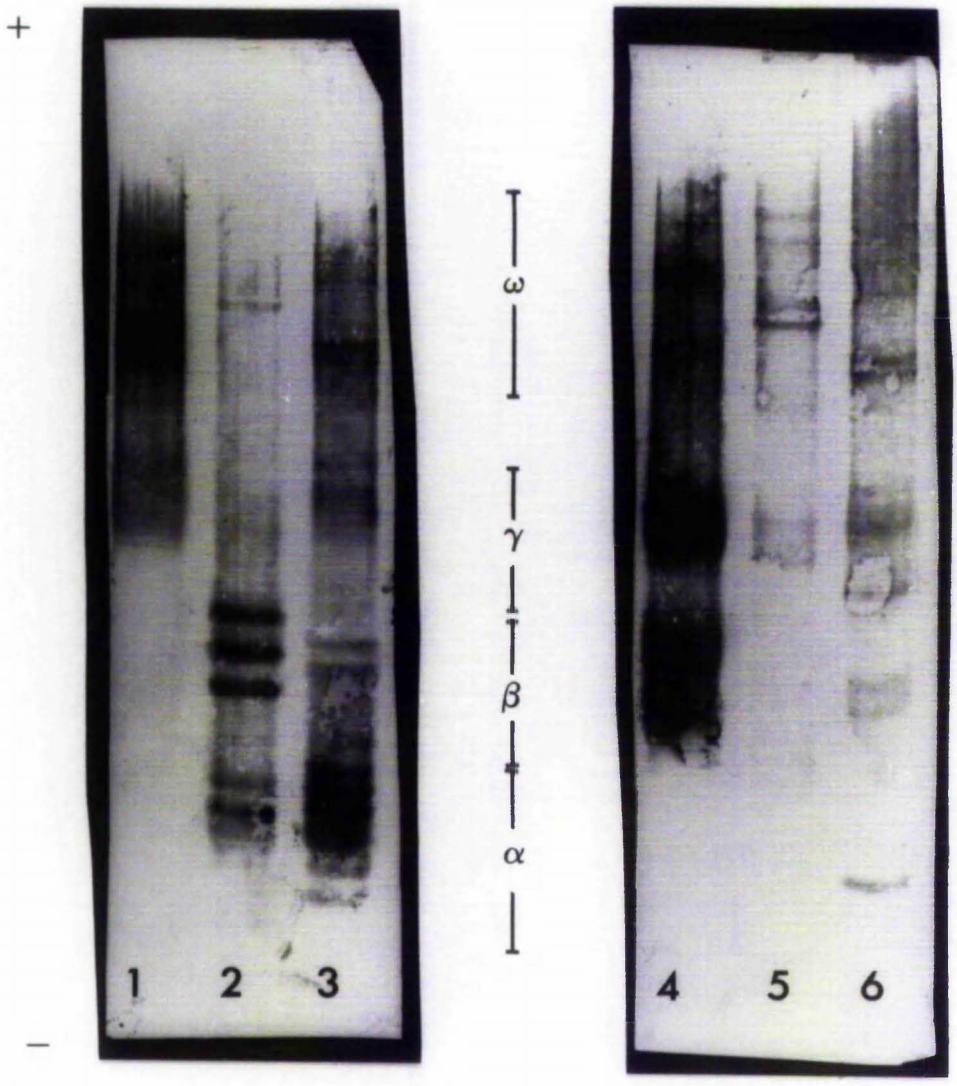
RP-HPLC fractionated γ/β-gliadins (lanes 1 and 4)

Avalon (lanes 2 and 5)

D. Valdur (lanes 3 and 6)

Lanes 1-3 were incubated with semi-purified test sera obtained from rabbits immunised with total Avalon gliadins. Lanes 4-6 were incubated with semi-purified test sera obtained from rabbits immunised with RP-HPLC fractionated γ/β-gliadins.

Western blotting and Immunoblotting were carried out according to the procedures outlined in sections 2.11 and 2.12.


+
1
2
3
ω
γ
β
α
4
5
6
−

3.4.2. Isolation of Common Wheat Specific Anti-Gliadin IgG by Indirect Affinity Purification

The objective of this section was to isolate hexaploid wheat specific anti-γ-gliadin IgG components from semi-purified (with albumin removed) rabbit anti-gliadin antisera. Most IgG molecules raised to hexaploid wheat gliadins recognised and bound to antigenic determinants on Durum wheat gliadins. However it was expected that some anti-gliadin IgG (raised to γ-gliadin) would not recognise Durum gliadin sites and would therefore specifically recognise only hexaploid wheat (Vu and Popineau, 1987).

3.4.2.1. Coupling of Durum Gliadins to Cn-Br Activated Sepharose 4B

Of the 25mg of Durum wheat gliadins incubated with 5ml of Cn-Br activated sepharose 4B, 24.5 mg of protein bound to the gel producing a binding efficiency of 98% and a gliadin capacity of 4.9mg per ml of gel.

3.4.2.2. Assessment of Antibody Affinity And Purification of Anti-γ-Gliadin

Before mixing the anti-gliadin anti-sera and the Durum gliadin bound sepharose 4B it was first necessary to estimate the antibody concentration required to saturate a known quantity of Durum wheat gliadins. A non-competitive ELISA was performed on serial dilutions (1/50-1/500,000) of the semi-purified antisera using "zero antigen" blanks. Antibody dilutions which correspond to the maximum absorbance obtained for 1μg of common wheat gliadin were taken as the anti-gliadin titer for the antisera.

For semi-purified antisera raised to total Avalon

gliadins 12.5 ml of a 1/100 dilution in PBS was used since 50μl of 1/10,000 dilution of this antisera was found to saturate 1 μg of gliadins. For semipurified antisera raised to RP-HPLC fractionated γ/β-gliadins 12.5 ml of 1/10 dilution in PBS was used since 50 μl of 1/1000 dilution of this antisera was found to saturate 1 μg of gliadins

Durum gliadin bound sepharose 4B (section 2.10.2.1) was then incubated separately overnight with semi-purified anti-Avalon gliadin antisera (1/100 dilution in PBS) and semi-purified anti-gliadin antisera (1/10 dilution in PBS) raised to RP-HPLC fractionated γ/β -gliadins (section 2.10.2.2.). After incubation, the gel and dilute antisera were separated by filtering through a glass scinter funnel. Bound IgG was chemically desorbed using 200mM Glycine HCl (section 2.10.2.3.) and both this solution and the filtrate were assayed (using immuno blotting) for cross-reactivity with hexaploid and Durum wheat gliadins.

3.4.2.3. Assessment of the Cross-reactivity of Affinity Purified Anti-gliadin IgG

Gliadin cross-reactivity was determined for both the filtrate and that of the desorbed anti-gliadin IgG prepared as described in section 3.4.2 from semipurified anti-gliadin antisera raised to RP-HPLC fractionated γ/β-gliadins. Dilutions (1/10) of these solutions were incubated with electrophoretically separated hexaploid wheat gliadins (Avalon and Mercia) and Durum wheat gliadins (D. Valdur) blotted onto nitrocellulose strips. After immunodevelopment the affinity purified filtrate contained anti-γ-gliadin IgG which recognised major and minor gliadin bands of protein

corresponding to γ-44 and γ-46 of the hexaploid wheat Avalon and Mercia (Figure 3.35 lanes 2 and 3 respectively). It has already been shown that for most common wheat these are heterogenous protein bands (section 3.3.1.2.) but it was not clear whether the major or minor gliadin protein was responsible for the antigenicity of γ-44 and γ-46. No cross-reactivity was observed between the affinity purified anti-γ-gliadin and D. Valdur gliadins (Figure 3.35 lane 1).

The anti-gliadin IgG desorbed from the affinity column recognised, β-, γ-, ω-gliadins of both hexaploid (Figure 3.35, lanes 5 and 6) and Durum wheat gliadins (Figure 3.35 lane 4).

In the following section the specificity of anti-γ-gliadin IgG affinity purified from antisera (Table 3.7, serum B) raised to RP-HPLC fractionated γ-/β-gliadins (as described in sections 2.10.2.2. and 3.4.2) was investigated by immunoblotting of electrophoretically separated gliadin extracts obtained from different varieties of Durum wheat, hexaploid wheat and pasta products.

3.4.2.4. Interspecies Cross-Reactivity of Anti-γ-Gliadin IgG

The cross-reactivity of affinity purified anti-γ-gliadin IgG was assessed against total gliadin extracts of the following wheat: 40 varieties of European hexaploid wheat, 5 varieties of European Durum wheat; 7 varieties of North American Durum wheat; 8 commercial pasta samples.

In 35 varieties of hexaploid wheat anti-γ-gliadin IgG raised to RP-HPLC fractionated gliadins cross-reacted with one or two of the γ-gliadins corresponding to 42, 44 or

Figure 3.35 Immunoblots Produced after Incubation of Western Blots of Total Gliadin Extracts with Affinity Purified Anti-gliadin IgG.

The samples illustrated are as follows:

D. Valdur (lanes 1 and 4)

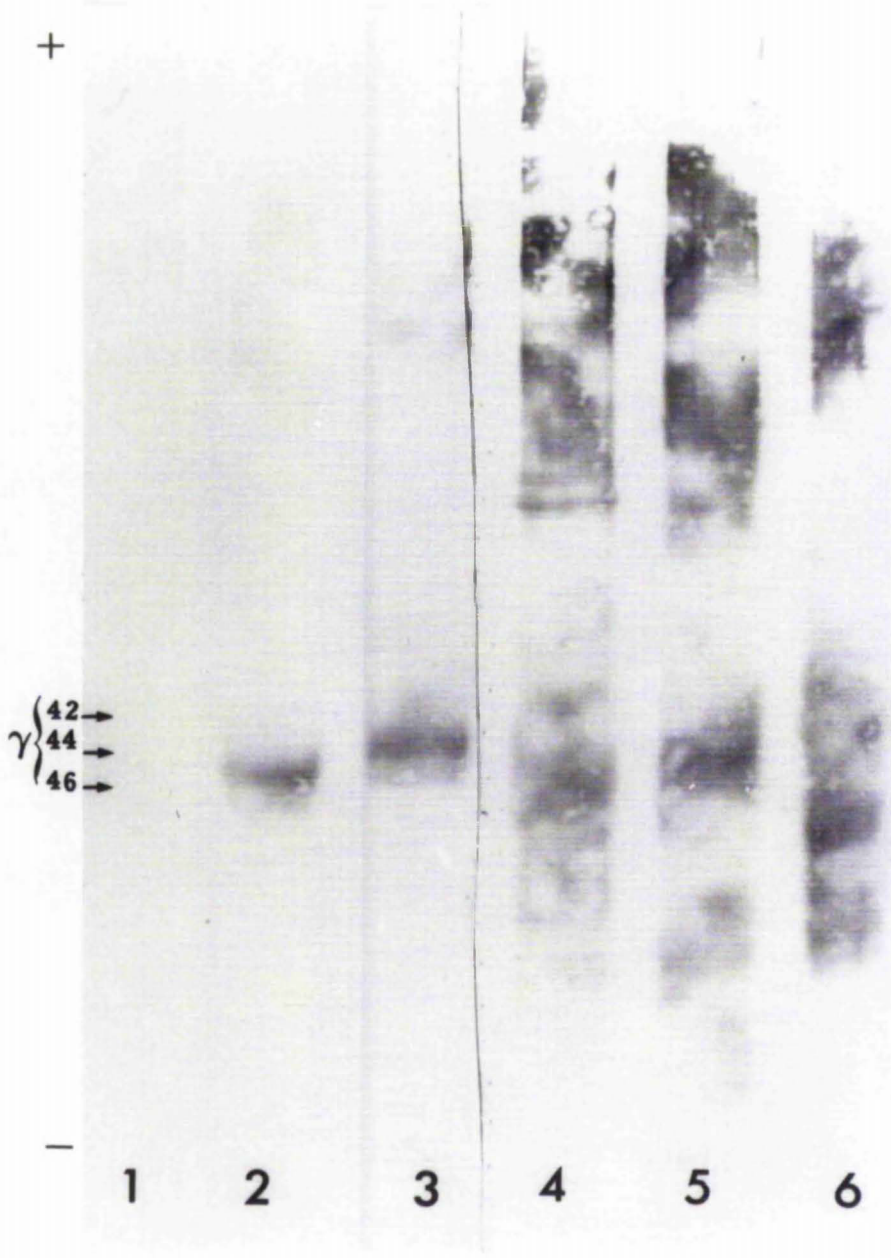
Avalon (lanes 2 and 5)

Mercia (lanes 3 and 6)

Lanes 1-3 were incubated with anti-gliadin IgG remaining after affinity purification (with Durum gliadin bound to sepharose 4B) of semi-purified test sera obtained from rabbits immunised with RP-HPLC fractionated γ-/β-gliadins.

Lanes 4-6 were incubated with the antibody preparation desorbed (after incubation with Durum gliadin bound to sepharose 4B) using 200mM Glycine-HCl at pH 2.5.

Western blotting and Immunoblotting were carried out according to the procedures outlined in sections 2.11 and 2.12.


+
γ
42
44
46
−
1
2
3
4
5
6

46. The immunoblot for 11 of these is shown in Figure 3.36. Selective variation was observed between the cross-reactivity of the anti-γ-gliadin IgG and each of these gliadins, furthermore the intensity of reaction varied significantly. Five varieties (Slejpner, Riband, Apollo, Norman and Longbow) were not recognised by the anti-gliadin IgG preparation. Immunoblots of gliadins from these varieties are shown along with Kanzler, Maris Huntsman, Sicco, Pernel and D. Valdur in Figure 3.37. It has already been shown (section 3.3.2.1) that gliadins corresponding to γ-42 , 44 and 47 were present on the 1D gliadin electrophoregrams of these varieties but these gliadins did not elute during RP-HPLC between 47-49 min as was seen for the majority of hexaploid wheat varieties (section 3.2.2). Furthermore γ-44 from Slejpner was shown in section 3.3.2.1. to be a homogenous band whereas in most other hexaploid wheat it was composed of two protein bands. It therefore appears that in Slejpner and probably in Riband, Apollo, Norman and Longbow either the epitopes on γ-44 gliadin or the minor gliadin band responsible for cross-reacting with anti-γ-gliadin IgG were either totally absent or present in very small doses which could not be detected by immunoblotting at the protein concentration present. However it was considered that the gliadin bands responsible for immunoreactivity were present at low levels as faint bands were observed in the γ-gliadin region of the immunoblot during colour development. However this band vanished after removal of the blot from the developing solution and after washing in water.

No cross-reactivity was found between the anti γ-

Figure 3.36 Immunoblots Produced after Incubation of Western Blots of Total Gliadin Extracts of European Hexaploid and Durum Wheat Varieties with Anti-γ-gliadin IgG.

The samples illustrated are as follows:

A. Brimstone, Galahad, Tonic, Alexandria, Brock, D Regal and D Capdur (lanes 1-7 respectively)

B. Avalon, D. Flodur, D. Arcour, Crossbow, Baron, Maris Dove, Hustler and Monza (lanes 1-8 respectively)

Western blotting and Immunoblotting were carried out according to the procedures outlined in Figure 3.35.

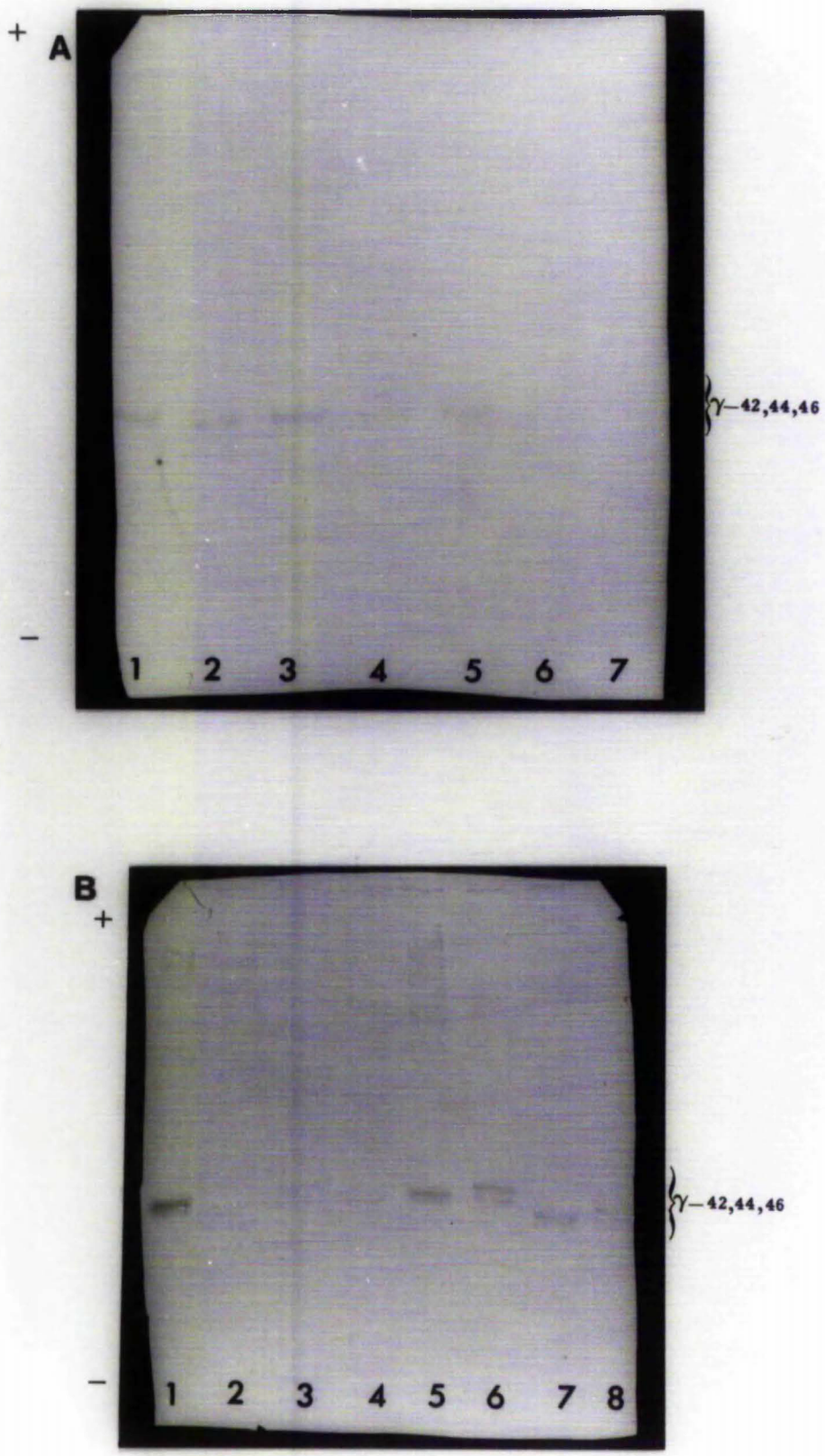
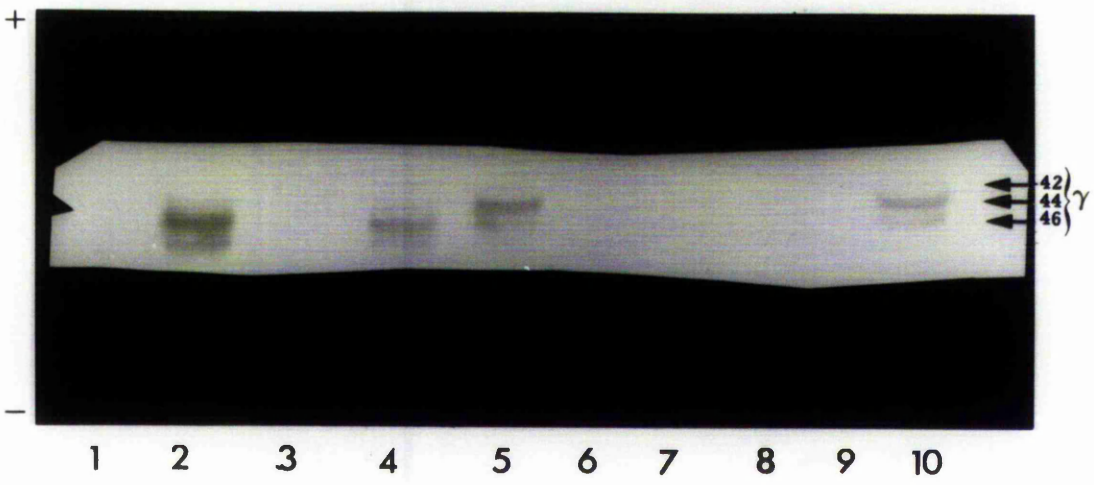

A
+
−
1 2 3 4 5 6 7
γ–42,44,46
B
+
−
1 2 3 4 5 6 7 8
γ–42,44,46

Figure 3.37 Immunoblots Produced after Incubation of Western Blots of Total Gliadin Extracts of 5 unusual European hexaploid wheat varieties with Anti-γ-gliadin IgG.

The samples illustrated are Slejpner, Riband, Apollo, Norman, Longbow (lanes 1,3,6,7,8,) which are not recognised by the anti-γ-gliadin antibody and Kanzler, Maris Huntsman, Sicco and Pernel (lanes 2,4,5,9) which are recognised by the antibody. D. Valdur is included in lane 10 as a control. Western blotting and Immunoblotting were carried out according to the procedures outlined in Figure 3.35.


+
−
42
44
46
γ
1
2
3
4
5
6
7
8
9
10

gliadin IgG and either European or North American Durum wheat gliadins. The resulting gliadin immunoblots for these varieties are presented along with those of hexaploid wheat varieties Figure 3.36 and Figure 3.38. In the gliadin immunoblots of the eight commercial pasta samples (Figure 3.39), anti-γ-gliadin cross-reactivity was observed in only two samples. Pastas 1-6 were apparently made from 100% Durum wheat and hence did not contain γ-44 and γ-46. For analysis of pasta three times the normal quantity of protein was electrophoresed. In all immunoblots, non-specific binding was observed, especially in the ω-gliadin region of the gel. However this effect was also observed when western blots were incubated with control antisera treated similarly. In terms of the detection of common wheat adulteration of Durum and pasta products the results obtained using immunoblotting complimented those obtained using conventional methods (TLC, electrophoresis and RP-HPLC).

3.4.2.5. Discussion

In developing the ELISA procedure to determine anti-gliadin antibody titers a gliadin extractant was needed which was efficient and yet maintained the antigenic integrity of the gliadin proteins to which the antibody bound. The detergent sodium dodecyl sulphate is a very good extractant of gluten protein (Bietz, 1984a) but the extracted protein can loose much of its antigenicity (Rizvi et al., 1980). Although alcoholic solutions are generally less effective extractants than 2M urea (Skerritt, 1985a) a 70% v/v aqueous ethanol solution was used to extract gliadin as it is suspected (Miflin et al., 1983) that urea inhibits the

Figure 3.38 Immunoblots Produced after Incubation of Western Blots of Total Gliadin Extracts of North American Durum Wheat varieties with Anti-γ-gliadin IgG.

The samples illustrated are Renville, Vic, Lloyd, Edmore, Quilafen, Ward and Wakooma (lanes 1-7) shown along with the hexaploid wheat variety Moulin (lane 8).
Western blotting and Immunoblotting were carried out according to the procedures outlined in Figure 3.35.

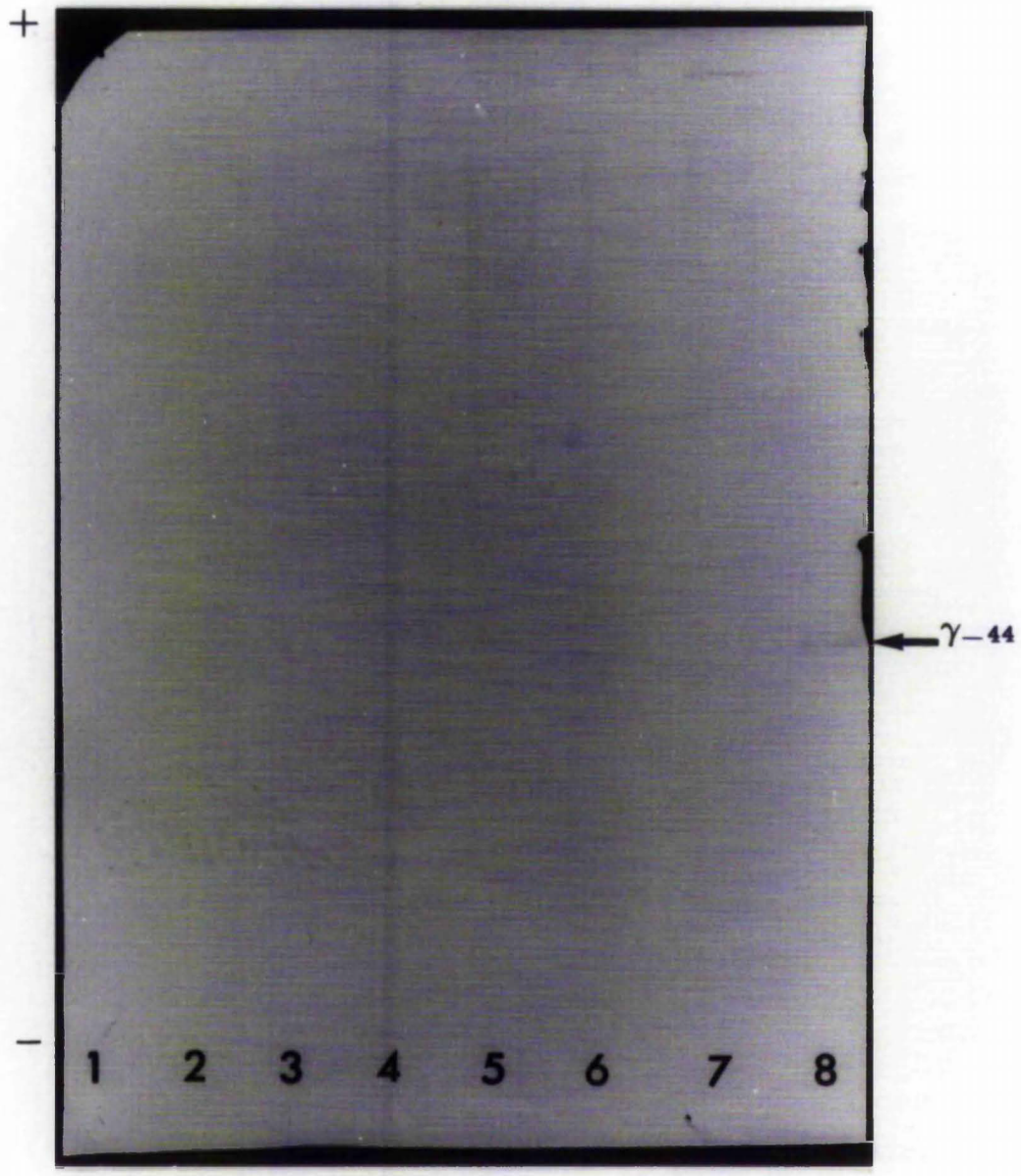
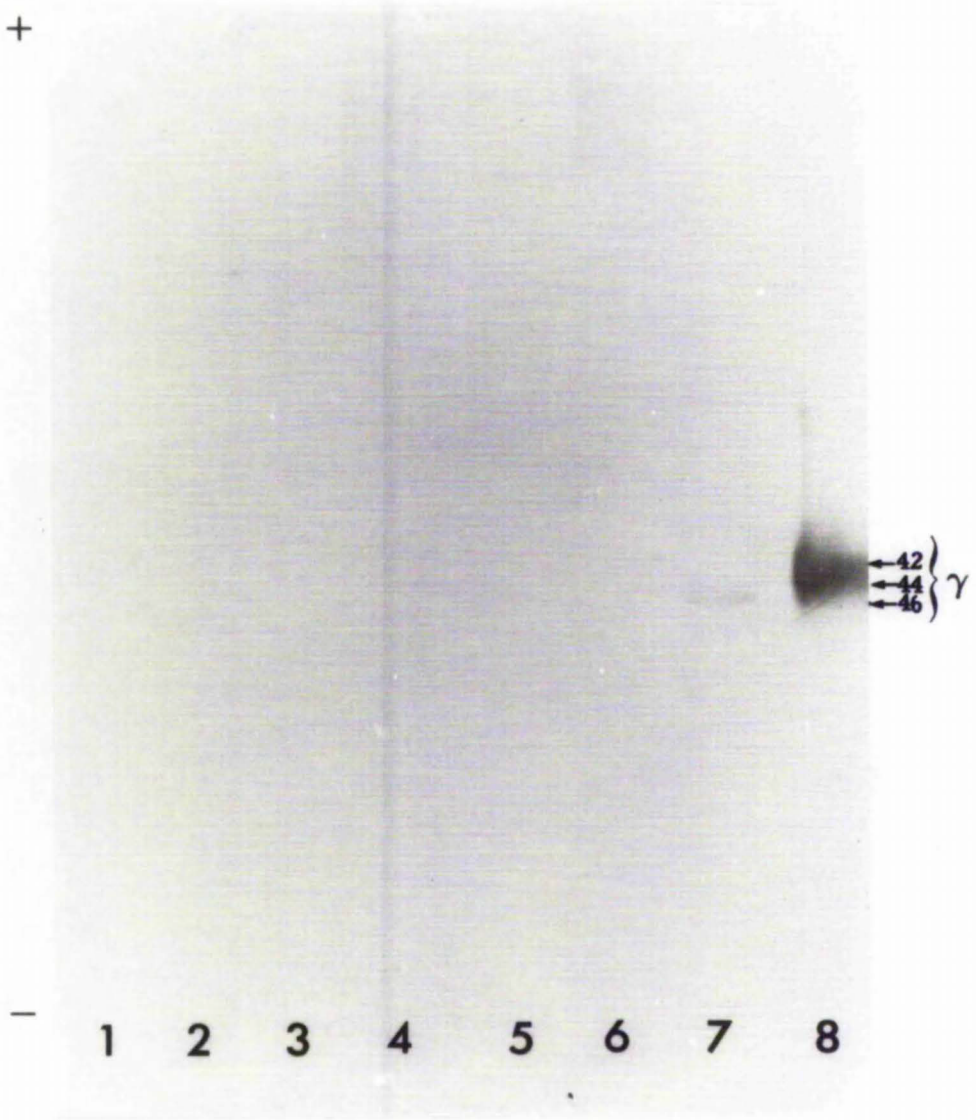

+
γ-44
−
1
2
3
4
5
6
7
8

Figure 3.39 Immunoblots Produced after Incubation of Western Blots of Total Gliadin Extracts of 8 pastas Samples with Anti-γ-gliadin IgG.

The pasta samples are illustrated in lanes 1-8. Anti-γ-gliadin IgG shows no reactivity with the pastas represented in lanes 1-6 but recognises a γ-gliadin band on the electrophoretic pattern of the pastas in lanes 7 and 8. Three times the normal volume (24μl) of gliadin pasta extract was applied to each well.

Western blotting and Immunoblotting were carried out according to the procedures outlined in Figure 3.35.


+
−
1
2
3
4
5
6
7
8
42
44
46
γ

antigen-antibody reaction. Skerritt (1985b) showed that the interaction of prolamins with polyvinyl carbonate is irregular and increases in the order rye > hexaploid wheat > Durum wheat > maize. However it was found that reproducible binding of both hexaploid and Durum wheat gliadins to the plastic microtiter plate was possible by precoating ELISA wells with poly-L-lysine/glutaraldehyde prior to adding the gliadin sample.

During the last five years knowledge of the composition and structure of wheat endosperm proteins has increased rapidly following the introduction of efficient separation methods (such as 2-dimensional electrophoresis and RP-HPLC) and the determination of amino acid sequences. Gluten subunits are now classified into seven types, ω-/γ-/β- and α-gliadins, high, medium and low molecular weight glutenins (Wieser et al., 1987). The components within these groups differ mainly by exchange, insertion and/or deletion of a few amino acids or small peptides resulting in proteins with only slightly different 1D electrophoretic mobilities at acid pH. Proteins having identical 1D electrophoretic mobility displayed heterogenity when separated by 2D electrophoresis and it was observed (section 3.3.2.1.) for a majority of hexaploid wheat that γ-44 and γ-46 are heterogenous protein.

The affinity purified anti-γ-gliadin IgG prepared in this study cross-reacted with either major or minor components of at least three gliadin bands (γ-42, 44, 46) as separated by 1D electrophoresis. It was not clear if these gliadins had common antigenic determinants or whether each

protein had a different antigenic determinant in which case more than one IgG was present in the affinity purified solution. It is still unclear if the antigenic proteins are present in the wheat cultivars Longbow, Riband, Slejpner, Norman, Apollo, since the gliadins in these varieties with mobilities corresponding to γ-42, γ-44 appeared to have different biochemical and immunochemical properties to the true γ-42 and γ-44 found in the majority of hexaploid wheat varieties. Antigenic determinants on γ-gliadins have been shown by Vu and Popineau (1987) to be different from those on α-/β-/ω-gliadins. In addition to this γ-44 and 46 were shown to have common antigenic determinants with each having its own unique antigenic structures.

The use of nitrocellulose for gliadin immobilisation in this study offered several advantages over more common means of studying antigen-antibody interactions such as double diffusion or immunoelectrophoresis in gels: water insoluble gliadins could be blotted directly onto the membrane after PAGE; the immune response could be easily amplified using the aviden-biotin peroxidase reaction, facilitating the detection of traces of γ-gliadin 44 or 46 which were present in a common wheat adulterated pasta; finally if rapid analysis is required it is possible to detect gliadins using a dot immunobinding assay (Skerritt and Smith, 1985).

4. GENERAL DISCUSSION

Since the initial introduction by Bietz (1983) of RP-HPLC separation of gliadins for the identification of wheat cultivars, many procedures applying this technique to solve complex problems in cereal chemistry have been proposed. The complexity of these applications has increased concomitantly with the development of sophisticated computer hardware and software which contribute to make gliadin RP-HPLC an automated, efficient, reliable and reproducible, analytical technique. In addition to computerised cultivar varietal identification (Scanlon et al., 1989a: 1989b) many studies have attempted to relate flour quality parameters to the RP-HPLC patterns of gliadins (Burnouf and Bietz, 1984a; Huebner and Bietz, 1986; 1987; Lookhart and Albers, 1988), LMM glutenins (Huebner and Bietz, 1985) and HMM glutenins (Huebner and Bietz, 1985; Lookhart and Albers, 1988; Sutton et al., 1989). Furthermore Burnouf and Bietz (1984a) showed that gliadin RP-HPLC profiles could be used to reliably predict the processing quality of Durum wheats. Genetic studies of wheat proteins using gliadin RP-HPLC profiles complimented 2D electrophoresis patterns showing that all gliadins are controlled by genes on the short arms of the group 1 and group 6 chromosomes (Burnouf and Bietz, 1984a; Bietz and Burnouf, 1985).

The first aim of this study was to demonstrate that RP-HPLC could be used qualitatively for variety identification of UK wheat and qualitatively for F1 hybrid purity analysis of wheat. Using gliadin RP-HPLC profiles, 18

UK wheat varieties were independently identified (Figure 3.2) on the basis of the presence or absence of particular peaks. The results were largely consistent with gliadin RP-HPLC results published for wheats grown in France (Burnouf et al., 1983), USA (Bietz et al., 1984), Canada (Kruger and Marchylo, 1985), Australia (Lookhart and Pomeranz, 1985), and New Zealand (Cressey, 1987). These workers reported that the majority of wheat varieties could be identified using RP-HPLC alone but a minority of wheats required further characterisation (using different techniques) to reveal their identity. One of the most notable features of these chromatograms was the reproducible nature of the "fingerprint" profiles obtained for each variety. Furthermore the elution times of peaks never differed by more than 25 seconds between runs.

The success of gliadin RP-HPLC as a method in cereal analytical chemistry depends on the chromatographic column used as well as the conditions under which proteins are eluted. Many workers (Marchylo and Kruger, 1984; Wingad et al., 1986; Marchylo et al., 1988) using optimised elution conditions suitable for each particular column showed that large pore size, reversed phase columns give the best results. Wingad et al. (1986) applied RP-HPLC columns with varying carbon chain lengths (C3-C8) and pore sizes of 150Å and 300Å for the separation of hordein protein. Also Marchylo and Kruger (1984) used a C18, 300Å pore size reversed phase column for the identification of barley cultivars. Both groups reported improved resolution when using columns with carbon chain lengths between 8-18 and

large pore sizes (300Å). Inefficient hordein binding was reported by Wingad et al. (1986) when using the 150Å pore size column. Similar results were obtained by Marchylo et al. (1988) for the analysis of wheat gliadins and these workers concluded that optimum resolution of gliadins occured using a Supercosil LC-308 column (C8, 300Å pore size, 5μm particle size, 5cm X 4.6mm id) at 50°C. In this study (using a Waters C18, 5-10μm particle size, 300Å pore size column) the resolution obtained for gliadin RP-HPLC elution profiles was equal to that obtained under similar conditions by Marchylo et al. (1988) who used a different extraction buffer (50% v/v aqueous propan-1-ol containing 4% DTT) a different column and a 120 min gradient.

Until the advent of RP-HPLC, acid PAGE of gliadins had been used almost exclusively for wheat variety identification. RP-HPLC resolves proteins primarily on the basis of differences in surface hydrophobicity, so the method compliments rather than duplicates, electrophoretic techniques that separate proteins on the basis of size and/or charge (Bietz, 1983,; Lookhart et al., 1986). Acid PAGE is also recommended for the detection of purity in hybrid wheat (Draper, 1987). The harvested F1 hybrid wheat is a complex mixture (containing more than one wheat variety) from which it is necessary to separately analyse between 100-400 seeds from each batch in order to get results which are statistically significant. This may become a major problem if the use of F1 hybrid wheat as a food crop increases since larger numbers of F1 hybrid seed would then need to be analysed. This would involve thousands of electrophoretic

separations and a considerable number of man hours. It was found in this study that peaks on the gliadin RP-HPLC elution profile reflect the genetic exchanges known to occur during the crossing of male and female genotypes. As a result of this an alternative method was proposed based on the quantitative RP-HPLC analysis of a bulk sample (100-400) of seeds from a given trial (section 3.1.2.3.). This approach provided reasonable accuracy (+/-5%) but estimations of the purity of seed samples of unknown purity still required considerable manual operation. Although computerised quantitative analysis of wheat admixtures has been proposed (Marchylo et al., 1988) further work is necessary to improve the normalisation of chromatogram peaks to improve prediction accuracy particularily at low admixture concentrations (< 5%) and to obtain an automatic procedure for quantifying admixtures (binary and more complex) over a wide range or admixture concentrations.

The success of quantitative RP-HPLC for predicting the purity of hybrid wheat depends on the analyst having advance knowledge of the parent wheat varieties from which the hybrid is bred. Contaminating wheat can usually be traced to inadequate sterilisation of the male endosperm in the "female" plants resulting in self-fertilised grains ripening and then being harvested with the F1 hybrid wheat crop (Rutz, 1987). Occasionally pollination of "female" wheat plants with wild type pollen can occur. This type of hybrid contamination is rare (Rutz, 1987) and can be detected by observing peaks on the F1 hybrid gliadin RP-HPLC profile not found on either parent.

There is little or no relationship between the electrophoretic behaviour of gliadin proteins and their sequence homology or surface hydrophobicity. Acid PAGE separates proteins in a gel matrix based on charge and mass difference and is useful for identifying individual polypeptides; but it greatly separates some peptides that have been shown to be closely related on the bases of N-terminal sequences and barely separates others that are more distantly related (Bietz et al., 1977). The technique therefore reveals little about gliadin protein relations. SDS-PAGE separates proteins primarily on the basis of their molecular size and also separates proteins which are closely related but fails to differentiate between proteins which are less closely related (Bietz et al., 1977). It is also interesting to note how closely gliadin electrophoretic mobilities relate to their amino acid compositions (Table 1.2). The content of glutamic acid and aspartic acid in gliadins is relatively constant, furthermore these residues exist mainly as glutamine and aspartamine. Thus, at acidic pH, the basic amino acid content primarily determines the electrophoretic mobility of the protein since the molecular sizes are similar. ω-gliadins contain 1.0-3.2% basic residues, γ-Gliadins contain 3.6-4.4% basic residues, β-gliadins contain 4.9% basic amino acids and α-gliadins contain 5.2-5.9% basic residues. Thus acid PAGE separation reflects the basic amino acid content of gliadin. However despite all its limitations acid-PAGE is still the chosen method in most cereal laboratories for cereal identification and determination of hybrid purity, This technique is also

widely used (especially in France) for the detection of common wheat adulteration of Durum wheat and pasta. Gliadin RP-HPLC offers many advantages over acid PAGE (section 1.3.5.), however both techniques compliment rather than duplicate each other and they can therefore be applied to similar samples giving more reliable results.

The second aim of this study was to demonstrate that RP-HPLC could be used qualitatively and quantitatively for detection of common wheat adulteration of Durum wheat (section 3.1.3.) and pasta products (section 3.2.). ω-Gliadins and β-/γ-gliadins were electrophoretically identified in early-eluting and late-eluting peaks in the gliadin RP-HPLC profiles of hexaploid wheat, similar protein was absent on gliadin RP-HPLC profiles of Durum wheat. It was shown (section 3.1.3.3.) that the presence of the late-eluting peaks on a Durum wheat profile was related qualitatively and quantitatively (down to 5 percent) to the level of common wheat adulteration of a Durum wheat or pasta. In addition it was found by combining acid PAGE and RP-HPLC that less than 5 percent common wheat adulteration could be detected if ω-or β-/γ-gliadins were identified by electrophoresis in the different peaks eluting on the gliadin RP-HPLC profile of an adulterated sample.

Although many methods (as outlined in section 1.1.6.3) had previously been proposed to detect common wheat adulteration of Durum wheat and pasta, natural variation in the composition of different wheat made many of these unsuitable. Furthermore it was shown in section 3.2 that most methods (with the possible exception of tlc of fatty

acid sterols and acid PAGE of gliadins) were unsuitable for application to pasta products dried at high temperatures under modern processing conditions. However gliadin proteins are composed of four groups of proteins with varying degrees of surface hydrophobicities (Popineau and Pineau, 1987) thus making them ideally suitable for easy and reproducable RP-HPLC. The protein is genetically pre-determined as a result of which it is not subject to changes in environmental conditions (Wrigley and Shepherd, 1973). Furthermore in section 3.2 it was shown that gliadin RP-HPLC profiles obtained for pasta processed at high temperatures were similar to those obtained for pasta processed at normal temperatures indicating that gliadin proteins are stable to high temperature processing.

The success of this RP-HPLC method (section 2.6.) to detect common wheat adulteration of Durum wheat and pasta products depends on all hexaploid wheats varieties having a major peak eluting between 47-49 min. This was in fact the case for the 40 varieties assayed in this study. Extensive variation was observed between the absolute peak areas calculated for the different varieties. Under normal circumstances these differences would create difficulties in quantifying the extent of adulteration. But as pasta is normally adulterated with a mixture of common wheat, the problem was circumvented by using standard wheat mixtures composed of different common wheat varieties.

A further discrepency which could detract from the use of this method is presented in section 3.3.1. It was shown that the gliadin RP-HPLC peak eluting between 47-49 min

for most common wheat varieties was composed of eight gliadin bands, γ-44, 46, 47 and β-54, 56, 57, 61 and 64. However five varieties were found in which this peak was composed of only five gliadin bands, β-50, 52, 56, 61 and 63. A major peak, similar to that found in the majority of common wheats was present in these varieties. But due to the absence of γ-gliadins in this peak the confirmatory test in which acid PAGE and RP-HPLC are combined could not detect common wheat adulteration by these varieties if based solely on the composition of the gliadin RP-HPLC peak eluting between 47 - 49 min. This emphasizes the need to collect both ω- and γ-/β-gliadin fractions when using this method.

Gliadins, γ-44, 46, 47 and β-54, 56, 57, 61 and 64 are unique to common wheat which makes them suitable as indicators of common wheat adulteration in Durum wheat and pasta. All these proteins are related to each other by having similarily high surface hydrophobicities. γ-Gliadins were previously shown (Popineau and Pineau, 1987) to have the highest surface hydrophobicity of all the gliadins. However it is now clear (section 3.1.3.2.) that some B-gliadins have similar hydrophobicity to γ-gliadins.

Bietz et al. (1977) while studying the N-terminal amino acid sequence of gliadin proteins first reported that some γ-gliadins had unique properties. They studied γ_{1-3}-, β_5- and α_{1-10}-gliadins isolated from ponca hard red winter wheat ($\gamma\text{-}_1$, $\gamma\text{-}_2$ and $\gamma\text{-}_3$ are equivalent to B-50, γ-46 and γ-44 respectively in this study, section 3.3.3.). Intepretation of their work revealed that γ-44 and γ-46 differed considerably in amino acid sequence from α- and β-gliadins.

γ-Gliadin 44 and 46 were found to be heterogenous proteins with considerable sequence homology in each fraction. In addition γ-44 and 46 were shown to be homologous to each other: 12 of their first 22 amino acid residues are identical (Table 4.1). Definite differences between γ-44 and 46 were also observed: γ-46 has only isoleucine at position 2, while γ-44 has methionine. Other major differences include residues 4 and 7 (glutamine present only in γ-44), residue 5 (glutamine only in γ-46), residues 8, 13 and 21 (leucine only present in γ-46) and residue 18 (glutamine and histidine in γ-44) (Table 4.1).

As a result of studying γ-gliadin extracted from the wheat varieties Ponca and Kolibri, Patey et al. (1975) first reported that major differences occur in the amino acid sequence of γ-44 and γ-46 extracted from different wheat varieties. The number of γ-gliadins found in different varieties was also found to vary. Kolibri γ-gliadin is apparently homogenous while Ponca contained at least five major γ-gliadins plus additional minor ones (Bietz et al., 1977). Chinese Spring contained two major γ-gliadins and four or five minor ones (Wrigley and Shepherd, 1973) and a flour milled from two wheat varieties (Meredith, 1967) contained at least nine major γ-gliadins. In addition to this it is now clear (section 3.3.1. and section 3.3.2.) that different γ-gliadin are found in Avalon and Slejpner. Avalon γ-44 and γ-46 as separated by 1D electrophoresis are heterogenous proteins on 2D electrophoresis each composed of one major and one minor band. But γ-46 is absent in Slejpner

Table 4.1. N-Terminal Sequences of Ponca Gliadins[a]

Gliadin	Residue Number 1 5 10 15 20 25
γ-46	N-I-G-V-D-P-W-G-Q-V-Q-W-L-P-Q-Q-Q-V-P-Q-L-Z-Q-P--Q- P Q V Q L I V Q L Q Q Q R Q
γ-44	N-M-G-V-D-P-W-G-Q-V-Q-W-V-P-Q-Q-L-Q-P-Q-Q-Q--Z--V-Q- P Q Q V Q Q L P Q Q H Q P

[a]Data are presented using the standard one letter notation for amino acids: D, aspartic acid; G, glycine; H, histidine; I, isoleucine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; V, valine; W, tryptophan; Z, glutamic acid. More than one amino acid in a position indicates heterogeneity (from Bietz et al., 1977).

and γ-44 is composed of one homogenous band of protein on 2D electrophoresis.

Bietz et al. (1977) showed that γ-44 and γ-46 differed in sequence from α- and β-gliadin but he proposed that α-, β-, and γ-gliadins exhibited homology as a group of proteins which probably originate from one or two ancestral genes. However more is now known about the genetics of wheat endosperm proteins which may help to provide an explanation for the variability seen in the type and composition of γ-gliadins extracted from different wheat varieties.

As previously outlined (1.1.2.) hexaploid wheat originated from three separate genomes or chromosome sets, all of which have a common precursor. The protein synthetic capacity must have greatly increased from combining these three similar sets of genes into hexaploid wheat. More importantly, in combination with this, gene duplication and nonlethal mutations at all ploidy levels have further increased the number of proteins synthesized. But of the very large number of gliadins possible in wheat most varieties appear to contain only a maximum of 40 gliadins as detected by 1D electrophoresis (Kasarda et al., 1976). Genes coding for endosperm storage proteins occur at nine complex loci on six different chromosomes (section 1.2.1.4.). On the short arms of chromosomes 1A, 1B and 1D and located towards the end are Gli-A1, Gli-B1 and Gli-D1, each of these loci carry three major gene families coding for ω-gliadins, γ-gliadins and LMM-glutenins. The remaining loci, Gli-A2, Gli-B2 and Gli-D2 occur near the ends of the short arms of chromosomes 6A, 6B and 6D respectively and code for α-, β-

gliadins and about two minor γ-gliadins (Table, 1.4). Payne et al. (1984b), Autran and Feillet (1975) have shown that Durum wheat viscoelasticity is related to genes coding for γ-gliadin 45 and ω-gliadin 35, known to be tightly linked to each other on the short arm of chromosome 1B and from technological studies propose that these are also linked with genes coding for LMM glutenin-2 which in turn is responsible for good quality in the wheat. Poor quality Durum wheat lacks this genetic combination. Furthermore Payne et al. (1984a) reported that Darius and the majority of wheat from eastern Nepal do not produce any proteins coded at the Gli-D1 locus: ω-gliadins, γ-gliadins, and LMM glutenins. It has been speculated (Payne et al., 1984a) that these differences are the result of deletions of very small pieces of chromosomes during crossing. Thus because storage protein genes are situated towards the end of the chromosome arm and are always clustered together at the same locus (Sozinov and Poperelya, 1980), linked genes could easily be lost during mutations.

This argument can be extended to explain the differences found between γ-gliadins 44 and 46 of hexaploid wheat varieties. Both the major and minor components of these gliadins are coded by either the 1D or 6B chromosome. Therefore in the varieties Slejpner, Riband, Apollo, Norman and Longbow due to a deletion mutation the gene encoding Y-46 could be totally absent from the chromosome resulting in the deletion of this protein. Furthermore it is also possible that the minor components of γ-44 and γ-46 are either completely absent or else are synthesised in insufficient quantities to facilitate their extraction in gliadin extracts

from Slejpner-type wheat. Even if extracted they are not detected by coomassie blue staining.

The results of the immunological study confirmed that homology exists between different γ-gliadin as it was apparent that up to 3 different 1D electrophoretically separated γ-gliadins (44, 46, 47) were recognised by the affinity purified antibody preparation. However the five hexaploid wheat varieties, Slejpner, Riband, Apollo, Norman and Longbow, were not recognised. γ-Gliadin 46, 44 minor and probably 46 minor were absent in these varieties. Thus if the minor gliadins are responsible for γ-gliadin immunospecificity seen in the majority of hexaploid wheat varieties its absence in Slejpner and Slejpner-type varieties explains the absence of immunoreactvity between these variety and anti-γ-gliadin IgG. On the other hand γ-44 (major) is present but not recognised by anti-γ-gliadin IgG. This supports the hypothesis that the minor gliadins are responsible for the selective antigenicity of γ-gliadin protein.

The differences observed in electrophoretic profiles of different common wheat varieties does not detract from the RP-HPLC method of common wheat adulteration detection in Durum wheat as this method is based on the detection of chromatographic peak, which are present in all the varieties studied. Neither does it alter to any great extent the operation of the RP-HPLC/electrophoresis method of adulteration detection as this is not totally reliant on the presence of the gliadins γ-44 and γ-46. However if the epitope responsible for the immunospecificity of γ-44 and γ-

46 are totally absent in a substantial amount of hexaploid wheat then it will make it very difficult to develop an immunochemical assay based on γ-gliadins alone. If however the observed differences are the result of deletion mutations then these are rare (Payne et al., 1984a) and should only effect a minority of wheat varieties. Alternatively the epitope may be more easily recognised if the gliadin extract was immobilised on a different support as quite different cross-reactivities were reported by Skerritt and Hill (1990) when the same gliadins extracts were used in indirect, antigen competition and sandwich type assay formats.

Ultimately an immunodiagnostic assay to detect common wheat adulteration of Durum wheat or pasta based on γ-44 or γ-46 is very desirable for the following reasons: gliadins are not influenced by changes in environmental conditions (Wrigley and Shepherd (1973); gliadins are encoded by either chromosome 1D or 6D (Payne et al., 1984a) which are absent in Durum wheat, consequently this anti-γ-gliadin IgG will not recognise Durum wheat γ-gliadin: and it has been demonstrated that anti-γ-gliadin will recognise γ-gliadin levels present in an adulterated pasta sample.

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Detection and Quantification of Adulteration of Durum Wheat Flour by Flour from Common Wheat Using Reverse Phase HPLC

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ABSTRACT

When common wheat (Triticum aestivum *L*) *gliadins were separated by RP–HPLC, a major doublet peak eluted at 47·20 and 47·94 min. This peak was consistently found to be absent in Durum wheat* (Triticum durum *Desf*) *gliadins separated under identical conditions. In Durum wheat gliadins a characteristic small peak eluted at 48·30 min followed at 50·47, 51·37, 52·80 min by larger peaks. The peak area ratio of the peaks eluting at 50·47 and 51·37 min was found to be 2·18 (±0·14). This ratio was found to decrease proportionally on contamination of Durum wheat flour with flour from some common wheat varieties. This ratio alone was not enough to detect and quantify adulteration by all varieties of common wheat. An alternative method was found whereby the peak emerging between 47 and 49 min in the Durum wheat gliadin elution profile was expressed as a ratio of the total protein applied. This ratio was shown to increase when Durum wheat flour was adulterated with flour from common wheat thus enabling quantitative estimation of the level of adulteration. A third method of detecting adulteration of Durum wheat flour is also proposed in which the peak emerging between 47 and 49 min is collected and the protein separated by PAGE. The presence of more than one band of γ/β-gliadins is indicative of adulteration.*

Key words: Reverse phase HPLC, adulteration, common wheat, Durum wheat, gliadins.

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NOTATION

RP–HPLC	reverse phase high performance liquid chromatography
PAGE	polyacrylamide gel electrophoresis
AUFS	absorbance units full scale

INTRODUCTION

A large proportion of European pasta products on sale are prepared from Durum wheat only. Italian law lays down rules for the manufacture and marketing of pasta products and prohibits the sale of products made from common wheat (*Triticum aestivum* L) or a mixture of common and Durum wheat (*Triticum durum* Desf). Extension of this prohibition to imported products has been ruled incompatible with Articles 30 and 36 of the EC treaty by the European court (C215/09, C215/10 1988). Owing to price differentials between types of wheats it is most likely that an increasing number of pasta products prepared from mixtures of Durum and common wheat will be traded.

For the correct application of import and export duty under the provisions of the Common Agricultural Policy of the European Communities it is necessary to differentiate between pasta products prepared solely from Durum wheat and those containing common wheat. For export refund purposes the actual proportion of common wheat has to be established. Pasta products purporting to be prepared from pure Durum wheat are commonly found in the marketplace. To enforce UK food legislation it is therefore necessary also to examine such products to check the validity of this claim.

Contamination of Durum wheat with common wheat varieties can occur during growing, harvesting or processing and may be accidental or intentional. Thus seed breeders, wheat merchants, pasta manufacturers and state quality control agencies need to have accurate and reliable methods for the identification of any possible adulteration of their wheat stocks. Currently Durum wheat flour (and pasta derived from it) is tested for adulteration with common wheat using one method or a combination of methods.

Electrophoretic methods are used whereby alcohol extractable proteins of the suspect sample are separated by gel electrophoresis and then examined for the presence of low mobility ω-gliadins (Resmini 1968; Kobrehel *et al* 1985; Dysseler *et al* 1986). Alternatively, water extractable proteins are separated by gel electrophoresis and the gel is examined for the presence of a specific polyphenoloxidase (Feillet and Kobrehel 1974). Water-soluble wheat proteins can also be separated by isoelectric focusing, and pure Durum wheat distinguished from common wheat by the absence of particular esterase bands (Cooke *et al* 1986). The electrophoretically identifiable moieties that distinguish the two species are normally only found in common wheat varieties, and their presence in Durum wheat is considered indicative of adulteration. The current EC recommended method for detection of adulteration of Durum wheat with common wheat involves identification of fatty acid sterol esters characteristic of common wheat, following

their extraction with petroleum ether and separation by thin-layer chromatography (Anon 1979).

Immunochemical methods using antisera to wheat globulin (Piazzi and Cantagalli 1969) and wheat albumin (Piazzi *et al* 1972) have also been reported. Where antibodies raised in rabbit and goat respectively have been used to assay for common wheat impurities in Durum wheat, levels of 10% and 5% have been detected using the wheat globulin antiserum and wheat albumin antiserum, respectively. However, the susceptibility of globulin and albumin cereal proteins to changes resulting from environmental growth conditions (Wrigley and Shepherd 1973) casts doubt on the reliability of such methods.

The two chromatographic methods quoted have been found to be accurate and complementary with remarkable reproducibility. However, the procedures are tedious and not easily automated, and interpretation is highly subjective. These methods are therefore used mainly as a qualitative assessment, any quantitative measurements being an estimate at best.

The application of RP–HPLC to protein separation has resulted in this technique being used in combination with electrophoresis as an analytical and preparative tool and, although these two techniques separate proteins by different criteria, Bietz (1983) and Lookhart *et al* (1986) have shown that the methods are highly complementary. RP–HPLC of alcohol-extractable proteins has been shown by several groups to be a useful technique for variety classification of barley (Marchylo and Kruger 1984; Marchylo and Kruger 1985; Wingad *et al* 1985, 1986), wheat (Bietz *et al* 1984; Huebner and Bietz 1987), rice (Lookhart *et al* 1987), oats (Lookhart 1985) and maize (Paulis and Bietz 1986).

Burnouf and Bietz (1984) have shown that RP–HPLC of gliadin proteins can be used to predict the gluten quality of Durum wheat varieties used for pasta manufacture, and Huebner and Bietz (1986) have found that it could also be used to assess the potential bread-making quality of hard wheats.

In this paper RP–HPLC is used to identify differences in aqueous 2-chloroethanol (250 mg ml^{-1}) extractable gliadins from varieties of common wheat and Durum wheat. These differences have been used to develop methods for detecting adulteration of Durum semolina flour with levels of common wheat flour as low as 50 g kg^{-1}.

MATERIALS AND METHODS

Glycine, acrylamide (Electran grade), N,N'-methylenebisacrylamide (Electran grade), ferrous sulphate, ascorbic acid, hydrogen peroxide, PAGE blue G-83 (Electran grade), acetonitrile (for HPLC 'far UV'), 2-chloroethanol (reagent grade, 99% pure), trifluoroacetic acid (Spectrosol grade), and methanol were supplied by BDH Limited, Poole, Dorset, Pyronin Y was supplied by the Sigma Chemical Company, St Louis, MO, USA. Acetic acid was supplied by FSA Laboratory supplies, Loughborough, Leics, and trichloroacetic acid (TCA) by the Aldrich Chemical Company, Gillingham, Dorset. All other reagents and chemicals used were of Analar grade, unless otherwise stated.

Wheat material

Seeds of European-bred Durum wheat cultivars (*Triticum durum* Desf) and European-bred common wheat cultivars (*Triticum aestivum* L) were obtained from authenticated stock held at the National Institute of Agricultural Botany, Cambridge, and the Plant Physiology Section, Trent Polytechnic Nottingham. North American-bred Durum wheat cultivars were received from The College of Agriculture, North Dakota State University, Fargo, North Dakota, USA.

The European-bred Durum wheat varieties used were Flodur, Capdur, Arcour, Valdur, Casour and Regal, and the North American-bred Durum wheat varieties used were Vic, Lloyd, Ward, Edmore, Renville and Quilafen. The European-bred common wheat varieties used were Avalon, Maris Dove, Crossbow, Rapier, Monza, Pernel, Sabre, Bounty, Baron, Mission, Sicco, Norman, Maris Huntsman, Alexandria, Brigand, Broom, Brimstone, Fenman, Jerico, Minaret, Kanzler, Hustler, Brock, Galahad, Hobbit, Mercia, Rendezvous, Solitare, Moulin, Hornet, Stetson, Slejpner, Parade, Riband, Tonic, Axona, Apollo and Pastiche.

Two mixtures (French, Belgian and English Durum) of 100% Durum semolina was received from the Laboratory of the Government Chemist, Teddington, Middlesex, and two commercial samples of Durum wheat semolina flour (one Greek and the other French) were obtained from Pasta Foods, Great Yarmouth, Norfolk.

Extraction procedures

Extractions were made with aqueous 2-chloroethanol (3:1 v). For electrophoresis, gliadins were extracted in the cold room for at least 2 h or, more usually, overnight, using 1 ml solvent per 250 mg milled wheat.

For RP–HPLC a solvent/milled wheat mixture (1 ml per 100 mg) was shaken vigorously in a flask shaker (Gallenkamp, Loughborough, Leics) for 2 min, placed in a cold room for 30 min and shaken for a further 1 min. Samples were clarified by centrifugation (12 000 × g, 10 min) and filtered through a 0·45-μm-pore filter (Waters Millipore, Harrow, Middlesex) before injection on to the column. Protein content was estimated according to the method of Lowry *et al* (1951).

Lactate polyacrylamide gel electrophoresis

Lactate PAGE was performed according to the method of Draper (1987) but the vertical slab gel apparatus supplied by LKB, Bromma, Sweden, was used. For optimum resolution it was essential to prerun gels at 500 V for 10 min prior to sample application. Electrophoresis was performed at a constant voltage of 480 V (current c 120 mA) for about 3 h at 18°C or for approximately twice the time taken for the pyronin Y marker to leave the gel, since the electrophoretic mobility of the marker is twice that of gliadin proteins. After fixing, staining and destaining of the proteins, R_m values were calculated relative to the position of an early eluting protein band common to all wheat gliadins (Sapirstein and Bushuk 1985).

Chromatography

All chromatography data were obtained using Waters Associates model 6000A and Waters Millipore model 510 solvent delivery systems and a Waters Millipore model

680 automated gradient controller. Samples were injected through a rheodyne 7000 injection valve connected to an LKB 2157 autosampler, and eluted proteins were monitored at 206 nm (0·4 AUFS) using an LKB 2238 Uvicord SII single-beam UV monitor. Fractions were collected for freeze drying using an LKB 2211 Superrac fraction collector. All water used was doubly distilled and purified using a Waters Millipore Milli-Q purification system to a conductivity of 0·05 μS. A Tecom C-100 water circulator supplied by Techne, Cambridge, was used to circulate water at 30°C to a glass column jacket in order to maintain constant column temperature during RP–HPLC. A Waters Delta Pak C18 5-μm particle-size (150 × 3·9 mm) reverse phase column was used when observation and quantification of differences were required, and a larger column (300 × 4 mm, Waters Delta Pak C18 10 μm particle size) was used when eluting proteins were collected for freeze drying and electrophoresis. A Magnus Scientific (Aylesbury, Buckinghamshire) Nucleosil-based C18 cartridge guard column, was installed between the injector and the column.

Data acquisition was by means of a Nelson Analytical (Warrington, Cheshire) 900 series interface box. Data manipulation and storage were facilitated by the use of a Walters (IBM clone) personal computer using Nelson Analytical software.

Elution gradients

A volume (20 μl) corresponding to 60 μg of protein was applied on the 5-μm particle-size column and a larger volume (200 μl) corresponding to 600 μg of protein was applied on the 10-μm particle-size column. Solvents A and B for elution of proteins consisted of aqueous acetonitrile 85:15 and 20:80 by volume, each containing 1 ml litre^{-1} TFA. A linear 45-min gradient from solvents A:B, ratio of 77:23 to 50:50 by volume with a hold at final conditions for 10 min and a flow rate of 0·5 ml min^{-1}, was used to elute proteins from the 10-μm particle-size column at a flow rate of 1 ml min^{-1} (Bietz *et al* 1984). After each analysis the column was washed with solvent B for 10 min before returning to the initial conditions.

Statistical analysis

Statistical analysis was carried out using the Minitab system (Minitab Inc, USA). The 95% confidence interval (CI) of sets of means was calculated and a two sample *t*-test was used to compare the difference between two means; when $P < 0{\cdot}05$ the difference was considered to be significant. The relation between several variables was measured by calculating the correlation coefficient between the variables. Mean values stated in the text are expressed as the mean $\pm$ SEM.

RESULTS

RP–HPLC of wheat gliadins

Wheat gliadins from the common wheat varieties Avalon and Maris Huntsman and the Durum wheat variety D Valdur were separated by RP–HPLC on a Waters Delta Pak, C18, reverse phase column (3·9 × 150 mm, 5-μm particle size, 300 Å pore size), as shown in Fig 1. The resulting profiles were overlayed and examined very

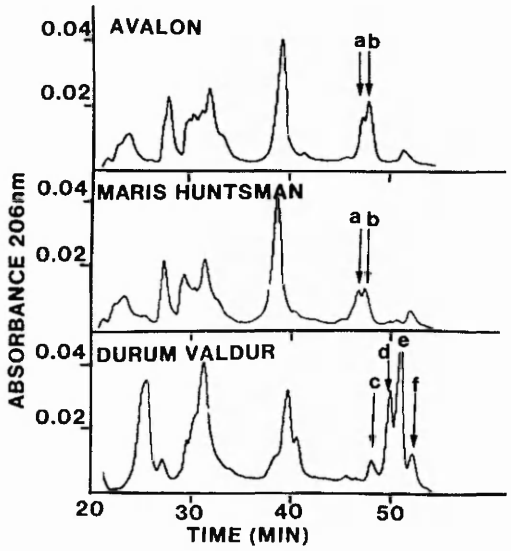

Fig 1. RP–HPLC separation of gliadins from common and Durum wheat varieties. Gliadin extracts (~60 μg of protein) were loaded on to a Waters Delta Pak, C18, 5-μm particle-size column (3·9 × 150 mm), and then eluted according to the procedure outlined in the Methods section. The profiles illustrated are Avalon, Maris Huntsman and D Valdur. Peaks at 47·31 and 47·93 min (peaks a and b) are characteristic of Avalon and Maris Huntsman and peaks at 48·73, 50·36, 51·30 and 52·80 min (peaks c, d, e, f) are characteristic of D Valdur.

closely over the entire profile using the chromatogram reprocessing facilities of the Nelson Analytical Data Processor. Two major peaks eluted on the RP–HPLC profiles of Avalon (Fig 1, peaks a and b) and Maris Huntsman (Fig 1, peaks a and b) at 47·31 and 47·93 min. Corresponding peaks at this time were absent from the D Valdur RP–HPLC profile. The gliadin RP–HPLC chromatograms from a further 36 varieties of European-bred common wheats (Maris Dove, Crossbow, Rapier, Monza, Pernel, Sabre, Bounty, Slejpner, Parade, Riband, Tonic, Axona, Pastiche, Apollo, Mission, Sicco, Norman, Maris Huntsman, Alexandria, Brigand, Brimstone, Fenman, Jerico, Minaret, Kanzler, Hustler, Galahad, Hobbit, Mercia, Rendezvous, Solitare, Brock, Broom, Hornet, Baron, Stetson) all had these two characteristic peaks eluting at 47·20 ± 0·33 min and 47·94 ± 0·05 min. The RP–HPLC profiles of six of these, Maris Dove, Crossbow, Monza, Pernel, Rapier and Maris Huntsman, are shown in Fig 2. In some varieties of common wheat (Bounty, Mission, Sicco) it was found that, in addition to the peaks eluting at 47·20 min and 47·94 min, two further major peaks eluted at 50·30 and 52·46 min (data not shown).

In the RP–HPLC profile of D Valdur (Fig 1) no peak was observed at 47·20 min or 47·94 min. A small peak (Fig 1, peak C) eluted at 48·73 min on the D Valdur gliadin profile which was not found in the common wheat profiles. Furthermore in the RP–HPLC profiles of D Valdur two large peaks (Fig 1, peaks d and e) eluted at 50·36 and 51·30 min. Further varieties of European-bred Durum wheat (Arcour, Flodur, Capdur, Casoar, Regal, Fig 3) examined under the same conditions all had a small peak eluting at 48·83 ± 0·08 min and two larger peaks eluting at 50·47 ± 0·10 min and 51·37 ± 0·07 min. A further small peak also eluted at 52·80 ± 0·07 min in all varieties (Fig 1, peak f). This characteristic pattern of peak elution was also found in the RP–HPLC profiles of the North American bred Durum wheat varieties (Vic, Lloyd, Ward, Edmore, Renville and Quilafen), in the RP–HPLC profiles of the semolina flour mixture (French, British and Belgian) obtained from the Laboratory of the Government Chemist and in the RP–HPLC profiles of the unidentified Greek and French semolina flour obtained from Pasta Foods.

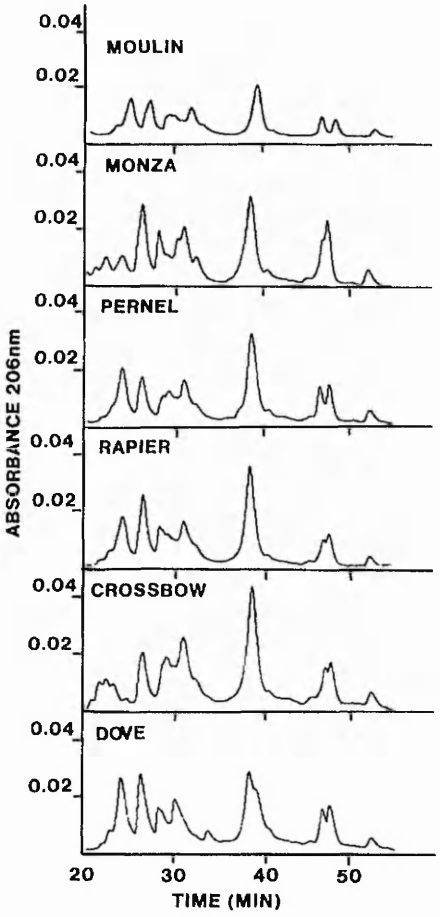

Fig 2. RP–HPLC separation of gliadin extracts from a number of different common wheat varieties illustrating the presence of the characteristic peaks denoted as a and b in Fig 1. Gliadin extracts (~60 μg of protein) were loaded on to a Waters Delta Pak C18, 5-μm particle-size column (3·9 × 150 mm), and then eluted according to the procedure outlined in the Methods section. The profiles illustrated are Moulin, Monza, Pernel, Rapier, Crossbow and Maris Dove.

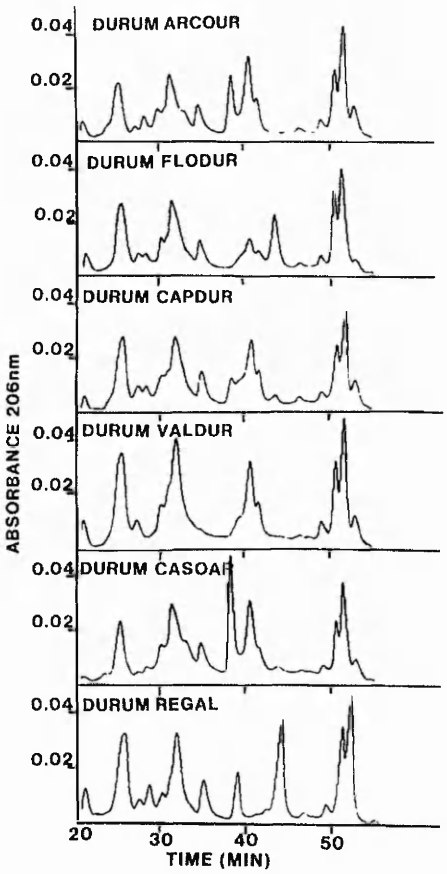

Fig 3. RP–HPLC separation of gliadin extracts from Durum wheat varieties illustrating the characteristic peaks eluting at 48·83, 50·47, 51·37 and 52·80 min. Gliadin extracts (~60 μg of protein) were loaded on to a Waters Delta Pak, C18, 5-μm particle-size column, and then eluted according to the procedure outlined in the Methods section. The profiles illustrated are D Arcour, D Flodur, D Capdur, D Valdur, D Casoar and D Regal.

Similar analyses carried out on the above Durum and common wheats, using the Waters Delta Pak, 10-μm particle-size column (4·6 × 300 mm, C18, 300 Å) gave identical gliadin profiles. Peak retention times varied slightly. The peaks eluting at 47·20, 47·94, 48·83, 50·47, 51·37 and 52·80 min on the Waters 5-μm particle-size column (3·9 × 150 mm) eluted at 48·90 ± 1·04, 49·50 ± 1·09, 49·75 ± 1·10, 51·40 ± 1·04, 52·40 ± 1·21 and 54·70 ± 1·21 min on the Waters 10-μm particle-size column (4·6 × 300 mm).

Electrophoresis of wheat gliadin RP–HPLC fractions

Fractions were collected from the RP–HPLC separation of Avalon, Maris Huntsman and D Valdur gliadins at 47·00–49·00 min and at 50·00–53·00 min. To

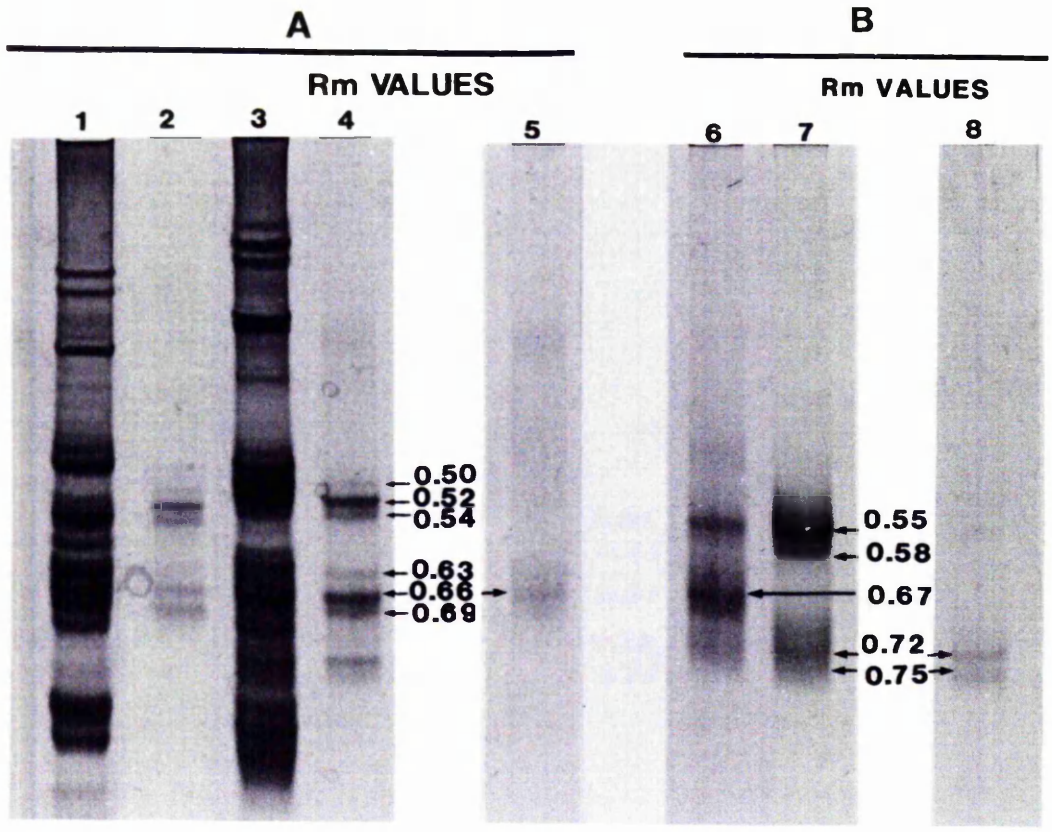

Fig 4. Electrophoretic separation of gliadins obtained from fractions collected following the RP–HPLC separation of total gliadin extracts from Avalon, Maris Huntsman and D Valdur using the chromatographic conditions outlined in Fig 1. The fractions illustrated are as follows. A: Lane 1, Total D Valdur gliadins; lane 2, Maris Huntsman fraction collected at 47–49 min; lane 3, Total Avalon gliadins; lane 4, Avalon fraction collected at 47–49 min; lane 5, D Valdur fraction collected at 47–49 min. B: Lane 6, D Valdur fraction collected at 50–51 min; lane 7, D Valdur fraction collected at 51–53 min; lane 8, Avalon fraction collected at 50–53 min.

enable collection of a sufficiently large sample the Waters Delta Pak, C18, 10-μm particle-size column was used. In the following text, retention times used to identify peaks eluting from the 5-μm particle-size column will continue to be used to identify peaks eluting from the 10-μm particle-size column to facilitate cross-reference.

Electrophoresis of the Avalon fraction collected between 47·00 and 49·00 min showed that it consisted of eight bands of protein in the γ/β-gliadin regions of the gel, with R_m values (Fig 4A) of 0·50, 0·52, 0·54, 0·63, 0·66, 0·69, 0·72 and 0·75. The last two bands were also found in the fraction eluting at 50·00–53·00 min (Fig 5B) and were therefore not considered specific to this RP–HPLC fraction. Electrophoresis of the Maris Huntsman fraction collected at 47·00–49·00 min showed that it consisted of six bands of protein in the γ/β-gliadin region of the gel, with R_m values (Fig 4A) of 0·50, 0·52, 0·54, 0·63, 0·66 and 0·69. Electrophoresis of the D Valdur fraction collected at 47·00–49·00 min (Fig 4A) showed that it consisted of one band of protein in the β-gliadin region of the gel with an R_m value (Fig 4A) of 0·66. Thus electrophoresis indicated that Avalon, Maris Huntsman and Durum have only one

common protein out of a possible total of six eluting at 47·00–49·00 min from the reverse phase column.

Electrophoresis of the D Valdur fraction collected at 50·00–51·00 min and 50·00–53·00 min (Fig 4B) showed that it consisted of five protein bands in the γ/β-gliadin region of the gel, with R_m values (Fig 4B) of 0·55, 0·58, 0·67, 0·72 and 0·75. No protein bands which had eluted in the previous fraction (47·00–49·00 min) were found in this fraction. Electrophoresis of the Avalon fraction collected at 50·00–53·00 min showed that it consisted of two protein bands in the β-gliadin region of the gel, with R_m values (Fig 4B) of 0·72 and 0·75. No definite protein bands eluted in this region of the Maris Huntsman profile.

Detection of adulteration of Durum wheat with RP–HPLC

Adulteration detection using peak area ratios

Initial methods for assaying the purity of Durum wheat flour centred on detecting changes arising from the ratio of the areas of two peaks eluting at 50·47 and 51·37 min (peaks e and f, Fig 1). The area ratio calculated for these two peaks for six Durum varieties and the two semolina flours (mixtures of English-, French- and Belgian-bred Durum varieties) gave a mean value of $2{\cdot}18 \pm 0{\cdot}14$ with a 95% confidence interval of 2·04–2·27. Following adulteration of Durum wheat flour with flour from the common wheat varieties Bounty, Mission and Sicco (varieties with peaks eluting at 50·30 and 52·46), it was found that this area ratio decreased in proportion to the level of common wheat present (0–500 g kg^{-1}). However, only adulteration at levels greater than 200 g kg^{-1} was detectable. Furthermore, when Durum wheat flour was adulterated with common wheat flour without peaks eluting at 50·30 and 52·46 min (Hobbit, Moulin, Avalon), adulteration could not be detected. A further method was therefore sought.

Adulteration detection using the ratio peak area/protein loading

The differences between the gliadin RP–HPLC elution profiles obtained from common wheat and Durum wheat species were further investigated as a basis for detecting and estimating the adulteration of Durum wheat flour with flour from common wheat varieties. The method developed involves separating a gliadin extract (of known protein concentration) of the suspect flour, by RP–HPLC, according to the procedure outlined in the methods section, and the region of the elution profile between 45·00 and 55·00 min is examined for the presence of peaks. If the applied sample contained only Durum gliadins, four peaks should be detected in this area eluting at 48·83, 50·47, 51·37 and 52·80 min (Fig 1). If the sample is adulterated, further peaks elute between 47·00 and 48·00 min (Fig 5). If the level of adulteration is severe (> 150 g kg^{-1}), the peak normally found at 48·83 becomes distorted and can sometimes cover the whole area between 47·00 and 49·00 min as a doublet peak. By expressing the entire integrated area eluting between 47·00 and 49·00 min as a ratio with the amount of protein applied to the column, a value is obtained which is found to be the maximum permitted in a sample claiming to be pure Durum. The actual ratio values obtained for six Durum varieties and two semolina flours are shown in Table 1. Statistically these values were found to have a mean of 561 ± 61, and a 95% confidence interval between 513 and 620. Thus an

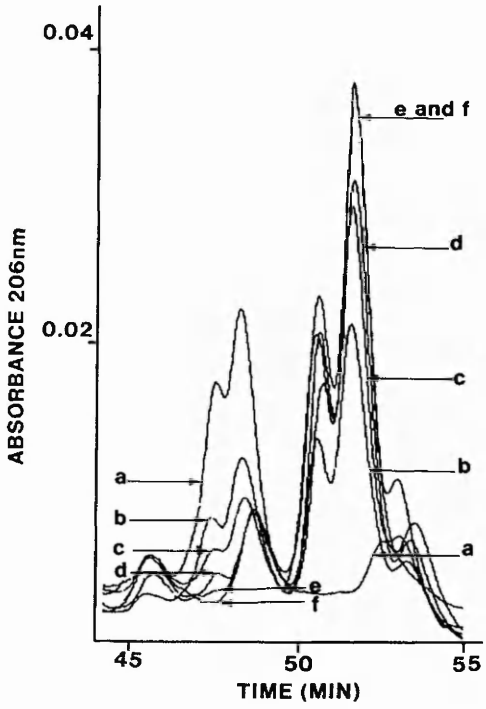

Fig 5. RP–HPLC separation of gliadin extracts from D Valdur illustrating the effect of adulteration (0–500 g kg^{-1}) with the common wheat Avalon on the elution profile between 45 and 55 min. Gliadin extracts (~60 μg of protein) were loaded on to a Waters Delta Pak C18, 5-μm particle-size column, and then eluted according to the procedure outlined in the Methods section. The profiles illustrated in overlay are (a) pure Avalon, (b) 500 g kg^{-1} Avalon in D Valdur, (c) 200 g kg^{-1} Avalon in D Valdur, (d) 100 g kg^{-1} Avalon in D Valdur, (e) 50 g kg^{-1} Avalon in D Valdur, (f), pure D Valdur.

adulterated sample with a level of adulteration greater than 5% (Table 1B, ratio = 602 ± 24) may be detected when the peak area/protein applied ratio is compared with that of the Durum standards (Table 1A).

To demonstrate this method, gliadins were extracted from a mixture of D Valdur adulterated with Avalon at levels of 50, 100, 150, 200, 300 and 500 g kg^{-1} and the extracts were separated by RP–HPLC. The resulting profiles are shown in an overlaid form in Fig 5. As the level of adulteration increases, a doublet peak emerges at 47·00–49·00 min; the apparent size of this peak increases as the level of adulteration increases. Adulteration at levels as low as 50 g kg^{-1} is clearly visible. Comparison of this with the gliadin electrophoretic pattern of the same samples (Fig 6) clearly highlights adulteration by the presence of the characteristic slow eluting ω-gliadins at 200 and 500 g kg^{-1} adulteration. At 100 g kg^{-1} these bands are barely detectable and it is expected from this test that adulteration levels less than 200 g kg^{-1} would be difficult to detect using electrophoresis alone.

A standard curve (Fig 7) was constructed relating the ratio of the peak (47·00–49·00 min) area to applied protein (Table 1B, correlation coefficient = 0·97) calculated for each Durum/common wheat mixture at the different levels (0–200 g kg^{-1}) of adulteration. As adulteration is usually caused by mixtures of common wheat rather than a single variety, the common wheat mixture chosen for use as adulterant was a blend containing 250 g kg^{-1} each of Avalon, Moulin, Maris Huntsman and Hobbit. The peak area ratios of unknown wheats can be compared with this curve in a manner similar to that when a normal standard curve is used, and a quantitative estimate can be obtained for levels of adulteration greater than 10% (Table 1B, $P > 0{\cdot}05$). Furthermore, visual detection is possible at adulteration levels as low as 5% (Fig 5). Thus normal practice in this laboratory is first to

TABLE 1

Relationship between area of peaks eluting between 47 and 49 min and total protein applied to an RP–HPLC column for (A) Gliadins extracted from flour from different Durum wheat varieties; (B) D Valdur adulterated with varying levels (50, 100, 150, 200, 300, 500 g kg^{-1}) of a common wheat mixture (composition 250 g kg^{-1} each of Avalon, Moulin, Maris Huntsman, Hobbit)

Wheat variety	*Area of peaks* $(a+b+c)$	
	Protein loading (μg) ($n=3$) *Mean* $\pm$ *SD*	
A		
D Capdur	558 ± 17	
D Flodur	592 ± 1	
D Valdur	580 ± 20	
D Casoar	462 ± 27	
D Arcour	480 ± 7	
D Regal	570 ± 40	
Semolina 1	615 ± 31	
Semolina 2	633 ± 33	
Mean $\pm$ SD	561 ± 61	
95% confidence interval	513 – 620	
B		
D Valdur + adulteration (g kg^{-1})		*P* values
0	580 ± 20	
50	602 ± 24	NS
100	655 ± 30	$P<0.050$
150	964 ± 11	$P<0.005$
200	1161 ± 59	$P<0.005$
300	2783 ± 194	$P<0.005$
500	4053 ± 157	$P<0.005$
Correlation coefficient of adulteration vs ratio = +0·97		

Ratios are calculated from protein loadings varying between 50 and 70 μg of protein (as outlined in the Methods section). Data represent the mean of three experiments.

calculate the internal ratio of the characteristic Durum doublet peaks eluting at 50·47 and 51·37 min, secondly to confirm the extent of adulteration by expressing the integrated area of the peak eluting between 47·00 and 49·00 min as a ratio with the total protein applied, and finally to quantify the extent of adulteration by reference to the standard curve shown in Fig 7.

Adulteration detection using electrophoresis following HPLC

If further confirmation of adulteration is required, a further method, combining RP–HPLC and PAGE, may be used for the detection of adulteration of Durum wheat with common wheat varieties. Having separated the gliadin extract on RP–

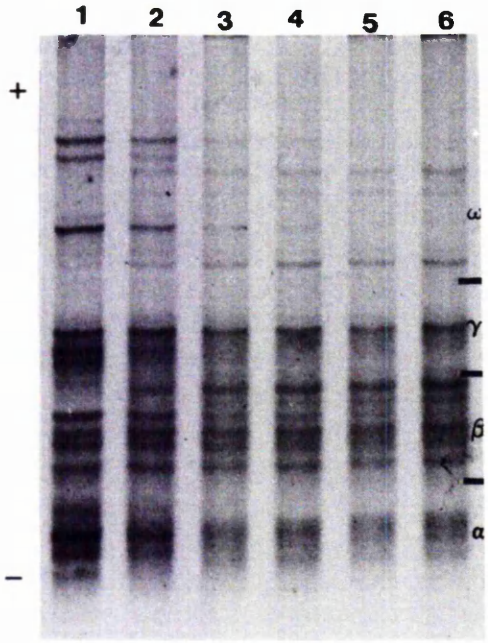

Fig 6. Electrophoretic separation of gliadins extracted from (1) pure Avalon, (2) 500 g kg^{-1} Avalon in D Valdur, (3) 200 g kg^{-1} Avalon in D Valdur, (4) 100 g kg^{-1} Avalon in D Valdur, (5) 50 g kg^{-1} Avalon in D Valdur, (6) pure D Valdur. Electrophoresis was carried out according to the method of Draper (1987) as outlined in the Methods section.

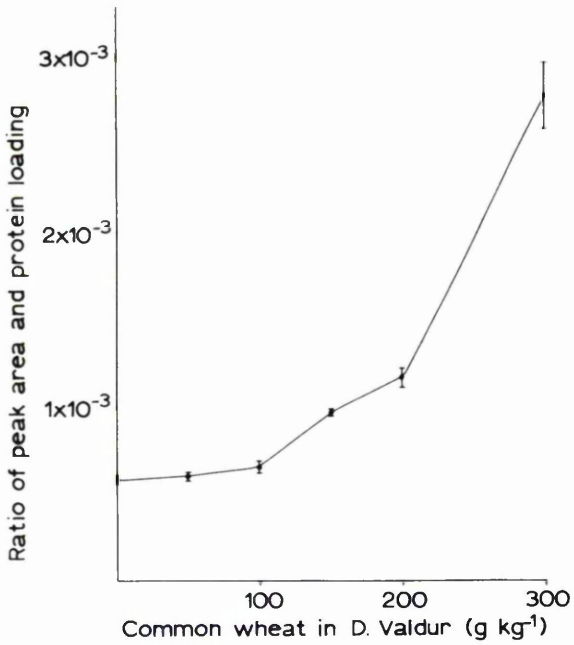

Fig 7. Standard curve showing the relationship between the increase in the ratio of the peaks eluting between 47 and 49 min and the total protein applied to the column, for extracts from pure D Valdur and D Valdur after adulteration (0–200 g kg^{-1}) with a common wheat mix containing the varieties Avalon, Moulin, Hobbit and Maris Huntsman.

HPLC, a fraction corresponding to the area at 47·00–49·00 min was collected and lyophilised. Thus all the protein in this area was concentrated facilitating further analysis of the protein in the peak using electrophoresis. If the sample was not adulterated, only one band of protein is detectable following separation by gel electrophoresis (Fig 4A, lane 5). This protein corresponds to the peak at 48·73 min

(Fig 1, peak d) on the gliadin RP–HPLC profile of pure Durum. If the sample has been adulterated, up to six bands of protein may be detectable (Fig 4A, lane 2) in the γ/β-gliadin region of the gel.

To quantify the level of adulteration using this method, the protein content of the gliadin extract applied to the column and the protein content of the RP–HPLC fraction collected at 47·00–49·00 min must be determined. It is then possible to relate the amount of protein in the peaks between 47·00 and 49·00 min with that present in the gliadin extract applied to the column and thereby calculate the level of adulteration for any unknown samples using unadulterated and adulterated controls as reference standards.

Although this method is sensitive down to adulteration levels of 50 g kg^{-1} and less, its use is limited by the fact that it is long and complicated and it requires a great deal of technical input. It is, however, suitable for detecting adulteration in samples where all other methods have failed.

DISCUSSION

When pure Durum wheat gliadins were separated under the conditions described, a characteristic small peak eluted at 48·83 ± 0·08 min (Fig 1, peak c) followed at 50·47 ± 0·10 min, 51·37 ± 0·07 min and 52·80 ± 0·07 min by larger characteristic peaks (Fig 1, peaks d, e, f). In comparison, when common wheat gliadins were separated under identical conditions, a major doublet peak eluted at 47·20 ± 0·33 min and 47·94 ± 0·05 min (Fig 1, peaks a and b respectively), and in three of the 38 varieties examined (Bounty, Mission and Sicco) these peaks were followed by peaks eluting at 50·30 and 52·46 min. These differences were used as the basis for a method to detect the level of adulteration of Durum wheat flour by common wheat flour.

The peaks eluting at 50·47 and 51·37 min on the Durum profile constitute a doublet peak. It was found that the mean ratio (e/d) of these two peaks taken from eight different Durum wheat varieties decreased on addition of common wheat varieties with major peaks eluting at 50·30 and 52·46 min. The decrease in this ratio was found to be proportional to the level of common wheat added. However, many Durum wheat flours may be adulterated with a common wheat whose elution profile will not have a peak at 52·46 min (eg Hobbit and Moulin) and hence the internal ratio value will not be altered.

The ratio of the integrated peak area at 47·00–49·00 min and total protein extract applied in a pure Durum wheat sample was found to be 561 ± 61 with a 95% confidence interval of 513–620 (Table 1). Samples with levels of adulteration greater than 50 g kg^{-1} may be detected and by reference to a standard curve (Fig 7) those adulterated by greater than 100 g kg^{-1} can be quantified when the peak area/applied protein ratios of the suspect gliadins are compared with the values obtained for peak area ratios of the standard mixtures of Durum and common wheats. The values (Table 1) used to construct this standard curve were the mean values taken from D Valdur samples adulterated with a sample of common wheat containing Avalon, Moulin, Maris Huntsman and Hobbit. Using a mixture reduces the error associated with the genetic diversity of common wheats, which causes differences in

the area of the peaks eluting between 47·00 and 49·00 min in common wheats. Furthermore, Durum wheat adulteration is usually caused by a variety of different common wheats, and again a standard mixture containing more than one variety of common wheat will give a more accurate representation of the level of adulteration.

It has been shown by Cooke *et al* (1986) and Metakovsky *et al* (1984) that the two wheat species can be distinguished from one another using lactate gel electrophoresis since Durum wheat lacks the prolamins encoded by the D-genome, most noticeably the ω-prolamin type believed to be controlled by genes on chromosome 1D. It was proposed by Kobrehel *et al* (1985) that this difference could be used as a basis for a test to detect adulteration of Durum wheat flour with common wheat flours, but no indication of the sensitivity of this method was given. In this study it has been shown that PAGE of total gliadin proteins at low pH values can detect adulteration of Durum wheat flour by common wheat flour at levels as low as $100\,g\,kg^{-1}$ (Fig 6). However, this method is not ideal for detecting adulteration at levels less than $200\,g\,kg^{-1}$ and is very subjective.

Electrophoretic separation of the protein in the fractions collected between 47·00 and 49·00 min on RP–HPLC of Durum and common wheat gliadins showed it to be composed of γ/β-gliadins (Fig 4A). It has previously been demonstrated by Cooke *et al* (1986) using electrophoresis that, along with the differences shown between common and Durum wheats in the ω-gliadin region of the gel, there are also some characteristic differences between the two species in the $\gamma/\beta/\alpha$-regions of the gel. It is suspected therefore that the proteins eluting between 47·00 and 49·00 min following RP–HPLC of common wheat gliadins (as outlined on Fig 1) are in fact proteins encoded by the D-genome.

RP–HPLC and electrophoresis may also be combined and used to detect and quantify the level of adulteration of Durum wheat by common wheats. The peak eluting at 47·00–50·00 min on the RP–HPLC profile of the suspect Durum flour gliadins is first collected and, after freeze drying, its protein is resolubilised with aqueous 2-cholorethanol and run on electrophoresis. If, after staining the gel, the protein is identified as γ/β-gliadin, shown in the present work to be present only in common wheat varieties, it is concluded that the Durum sample is adulterated. A quantitative result can be obtained if the protein content of the initial extract and the peaks eluting between 47·00 and 49·00 min is known. Although more tedious, this method is highly sensitive since concentration of the adulterant proteins is facilitated prior to electrophoresis.

In conclusion RP–HPLC separation of aqueous 2-chloroethanol ($250\,mg\,ml^{-1}$) extractable gliadins proved a reliable way of detecting and quantifying adulteration of Durum wheat flour with common wheat flour. A large number of the wheat varieties used in this study were of UK origin. But studies here using Durum wheat obtained from North America and France and semolina flour obtained from Greece, Belgium and France, combined with common wheat from Germany, Sweden, Holland and France, suggest that the method may have the potential for much wider use than in the UK and the European Community. Its main advantages is increased sensitivity and its ability to produce a quantitative result within 90 min of receipt of sample. Lactate PAGE of gliadins requires up to 5 h, and TLC of fatty acid sterols can take up to 3 days. The application of this method to heated semolina products is currently under investigation.

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WHEAT VARIETY IDENTIFICATION SYMPOSIUM

Chorleywood, Thursday, 24 November

Chairman: Dr S C W Hook, Head of Milling Section, FMBRA

Programme

9.15 Registration and coffee

9.45 *Modern methods of wheat variety identification:* Dr R J Cooke, Head of Biochemistry Section, National Institute of Agricultural Botany

10.30 *Visual examination of wheat grains as an aid to variety identification:* Mr B A Stewart, Head of Wheat and Flour Testing Section, FMBRA

11.15 Coffee

11.30 *Wheat variety checking by image analysis: current results and thinking:* Dr P D Keefe, Technical Development Officer, National Institute of Agricultural Botany

12.15 *The application of HPLC in the identification of cereal cultivars:* Mr K McCarthy and Dr M Griffin, Dept of Life Sciences, Trent Polytechnic

1-2.00 Lunch

2.00 *Wheat variety identification by electrophoresis:* Mrs S E Salmon, Senior Scientist, Wheat and Flour Testing Section, FMBRA

2.45 Tea

3.00 *The application of electrophoresis to grain intake at UK flour mills:* Mr J Shine, Quality Control Executive, RHM Southampton

3.45 General discussion