

Measurement and Testing Programme (BCR)

Determination of common wheat in pasta products dried at elevated temperatures

**Final report of the contract no. 5266/1/5/333/89/10-BCR-F(10)
(1990-1993)**

**by J.C. Autran, P. Barnwell, M. Bony, A. Landi, I. Lumley, M. Griffin, A.
Paraf, P. Resmini, W. Stimson, P. Violle**

15 February 1994

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Economic Background

Pasta are important foods in Europe, mainly in Italy (25.8 kg/capita/year), France (6.6 kg), UK (4.8 kg), Germany (4.5 kg) and Spain (4.3 kg). The best products (nutritional value, cooking quality, aspect) are processed from durum wheat semolina. Because of this difference in quality, and because of the new market regulation on January 1993, that includes a free circulation between the member States of pastas made of durum wheat / common wheat mixtures, the consumers have to know through an efficient and verifiable labelling the exact composition of the pastas that are marketed.

In the past years, several methods were used for this purpose, especially electrophoretic or immunochemical determinations of proteins (albumins) or enzymes (peroxidases) specific of the genome D of common wheats. All these methods, however, are no longer reliable because of the introduction of technologies of pasta drying at elevated temperatures or of the development of new products (precooked pasta).

Therefore, one or several other methods to determine the relative amounts of durum wheat and common wheat used in pasta manufacture had to be urgently developed that are compatible with the use of elevated temperatures in the pasta industries. It is the reason why the Commission of the European Communities (Community Bureau of Reference, DG XII) supported a four-year research programme (1990-1993) in view to explore, and to test through inter comparisons, several possible methods.

All these new methods had also to be based on protein components that are specific of the genome D of common wheats, but, unlike the previous methods, they had to involve heat-resistant components, or at least take into account the denaturing effects of the new technologies. Furthermore, as a result of the tendency to decrease the price difference between common wheat and durum wheat (and assuming that the admitted limit for common wheat in high quality pasta products will be established at 2%), emphasis had to be put on the 0-5% common wheat range.

Initial Aims of the Project

- (i) To develop a method (or methods) capable of detecting and quantifying the presence of common wheat in durum pasta products dried at elevated temperatures.
- (ii) The method should permit the detection of common wheat flour at concentrations in the 0-5% common wheat range.
- (iii) The method should be independent of the drying protocol used for the pasta.
- (iv) The method should be independent of the genetic variation found within common wheats.

Initial Methods Proposed

1. Electrophoresis of and quantitation of 1D- ω -gliadins
Technologie des Céréales INRA, Montpellier, France (J.C. Autran)
2. Reversed-phase high-performance liquid chromatography (RP-HPLC) and immunodetection of specific γ -gliadins
Life Science, Nottingham Polytechnic, Nottingham, UK
(M. Griffin)
3. Immunochemical detection of common wheat specific albumins (Mb 0.28)
Immunologie INRA, Nouzilly, France, and
Technologie des Céréales INRA, Montpellier, France (P. Violle/A. Paraf)
4. Determination of specific albumins ("A", "B", "C") of common wheat
Università di Milano, Italy (P Resmini)
5. Durum Test Immunoassay on *Friabilin*
University of Strathclyde, and
Rhône-Poulenc Diagnostics, Glasgow, UK (M. Bony/W. Stimson)

**DEVELOPMENT OF METHODS TO DETECT ADULTERATION
OF DURUM WHEAT PASTA PRODUCTS WITH COMMON WHEAT**

Final Report of the Contrat MATI-CT-940015 (Standards, Measurement and Testing Programme 1995-1998), 164p.

Partners in the Project and Authors of the Final Report:

J.-C. Autran ¹, U. Bersellini ², M. Bony ³, M. Griffin ⁴, B. Gibbons ⁴, I.D. Lumley ⁵, I. De Noni ⁶, C. Profilis ⁷, P. Resmini ⁶, W.H. Stimson ⁸

1. INRA, Montpellier, France
2. Barilla Alimentare S.p.A. Parma, Italy
3. Rhone diagnostics technologies, St Didier Au Mont D'or, France
4. Nottingham Trent University, Nottingham, UK
5. Laboratory of the Government Chemist, Teddington, UK (Project Co-ordinator)
6. DISTAM, University of Milan, Italy
7. Standards, Measurement and Testing Programme, DG XII, Brussels, Belgium
8. University of Strathclyde, Glasgow, UK

Executive Summary

- A range of pasta samples with known common wheat content and processing history have been prepared, and have been used for method development and validation studies, and for a methods intercomparison. This critical part of the project highlighted the requirement for 'standard' pastas, i.e. pasta samples with known composition and thermal history.
- An SDS-PAGE procedure has been developed which clearly separates common wheat ω -gliadins from other proteins extracted from pastas. This method shows advantages over the previously published A-PAGE procedure. A recommended assay protocol is included in this report.
- An HPLC procedure has been developed which provides greatly improved resolution of common wheat ω -gliadins from other proteins extracted from pastas. The target peaks elute early in the chromatogram and are easily identified. A recommended assay protocol is included in this report.
- Monoclonal antibodies to D-genome gliadins have been produced and an ELISA has been developed which can be used to detect the presence of common wheat in Durum wheat pastas dried at elevated temperatures. A recommended assay protocol is included in this report.
- Work has been undertaken to investigate the formation of Maillard Reaction (MR) products in pasta during production processing to assess whether furosine and lysylpyrrolaldehyde could be used as indicators of pasta thermal history. Mathematical and graphical relationships are presented which describe MR product formation. Recommended assay protocols for the determination of MR products are included in this report.
- Work has been performed to assess protein extractability from pastas dried at different temperatures using dye-binding and Kjeldahl procedures. Information obtained suggests that these procedures can be used to classify pastas according to their thermal history and to normalise results to account for such differences. A recommended assay protocol is included in this report.
- A polyclonal antibody against the common wheat protein Friabilin has been prepared and a sandwich ELISA developed using the polyclonal antibody as the capture antibody and the F7F monoclonal antibody for recognition. The developed assay is not susceptible to interferences from food ingredients, such as egg and spinach, which affected the ELISA designed on only the F7F monoclonal.
- A revised assay format has been developed for screening pasta samples using a membrane strip assay and the F7F monoclonal antibody (against Friabilin) labelled with alkaline phosphatase for recognition. The screening test

is rapid (1 hour) and only simple apparatus is required. A recommended assay protocol is included in this report.

- A methods intercomparison has been held to evaluate developed methods of analysis. The results have shown that two or more of the developed methods of analysis, when used in concert, will provide reliable complementary evidence regarding the common wheat content of supposed Durum pasta samples.

1. Introduction and Background

Pasta has traditionally been produced from Durum wheat (*Triticum durum*) because it produces a better quality product than common wheat (*Triticum aestivum*). If significant amounts of common wheat are present in pasta, adhesion of gluten proteins to starch granules is hindered which leads to loss of starch on cooking and results in pasta with a 'sticky' surface. Common wheat as traded is cheaper than Durum wheat; this price differential (currently 110 ECU per tonne) may provide some suppliers of raw material and manufacturers with an incentive to fraudulently benefit by the undeclared addition of common wheat to supposed 100% Durum wheat pastas. In addition, at the time the project commenced Durum wheat attracted a subsidy of approximately 280 million ECU per annum; an additional incentive for the dishonest trader/manufacturer to adulterate with common wheat.

National legislation in 3 EU member states prohibits the manufacture of pasta containing common wheat (*Triticum aestivum*) for sale in their own countries, but not for subsequent export. In addition, Community food legislation prescribes that foods must not be misdescribed or presented or labelled in a misleading manner, thus pasta products labelled as being prepared from 100% Durum wheat must not contain common wheat. There is a caveat to this last statement however; Durum wheat (*Triticum durum*) pasta for export outside of the European Union is allowed to contain up to 3% (m/m) of common wheat which represents the maximum amount acceptable from unavoidable adventitious contamination during harvesting and transport etc. (EC Commission Regulation 1222/94). This 3% limit is considered to be the acceptable maximum for trade and enforcement issues.

In 1994 the EU production cost of pasta was 4.6 billion ECU, with an export value of 403 million ECU, and a production volume of 6.9 million tonnes. Italy produces over 70% of European pasta. Pasta is an important food in Europe. Italy has the highest per capita consumption (28 Kg per year), followed by Greece (8.9 Kg), Portugal (7 Kg), France (6.8 Kg), UK (4.8 Kg), Germany (4.5 Kg) and Spain (4.3 Kg). High growth rates in pasta consumption are predicted because consumers consider it to be a healthy (low in fat, sugar and added salt, high in carbohydrate and fibre), cheap and an attractive and variable alternative to traditional dishes.

From the above it is clear that there is a need for analytical methods which can detect and determine the amount of common wheat in Durum wheat pasta products, taking account of the 3% allowed for adventitious contamination.

Durum wheat is older than common wheat in evolutionary terms. It is a tetraploid wheat which evolved from the fusion of two wild grasses and contains two similar sets of seven chromosome pairs which are known as the A and B genomes. Common wheat is hexaploid with a third set of chromosomes known as the D genome. The D genome is responsible for the expression of a range of proteins

which occur in common wheat but not in Durum wheat, thus providing target analytes for the detection of the presence of *Triticum aestivum*.

Historically methods used to detect the presence of common wheat in Durum wheat pastas included lipid (sterol) analysis, and the electrophoretic and immunological determination of proteins (albumins), or enzymes (peroxidases) which were specific to the D-genome of common wheat. Sterol analysis is particularly insensitive and it has been shown not to be a reliable indicator of the presence of common wheat. The introduction of high-temperature drying of pasta products (temperatures greater than 60°C) rendered the traditional protein based methods inappropriate and unreliable because the target proteins were denatured, and assay sensitivity decreased or was completely nullified. Hence alternative analytical procedures had to be developed

The EU Commission funded a project within its Measurements and Testing Programme (BCR) (1990-1993) to develop methods capable of detecting and quantifying the presence of common wheat in durum pasta products dried at elevated temperatures. A report of this project was published in 1994 (Report EUR 16070 EN). The conclusions were that if pasta samples of unknown thermal history were examined there was no one procedure that could be proposed as a reference method for the European Union. However if samples were examined using a combination of electrophoresis for the detection of ω -gliadins, HPLC for the detection of γ -gliadins and immunoassay for the detection of friabilin, then useful conclusions could be made regarding Durum pasta authenticity. The report recommended that further research and developmental work was required to achieve the original objectives of the project.

The current project (EU Contract No MATI-CT 940015) had the aim of continuing the method development and validation studies. The stated primary objective was to develop and validate electrophoretic, HPLC and immunoassay procedures capable of detecting common wheat varieties in Durum wheat pasta products which had been dried at elevated temperatures. The methods to be developed were to have a detection limit of less than 30g/Kg of common wheat in Durum wheat products.

The work programme was designed to consist of the following work packages:

- development of simplified electrophoretic detection of common wheat ω -gliadins;
- refinement of a high performance liquid chromatographic procedure to determine common wheat ω -gliadins;
- improvement of an immunoassay to detect common wheat D-genome encoded proteins; improvement of immunoassays to detect Friabilin;

- assessment of the effects of non-wheat proteins on analytical procedures; evaluation of the thermal history of pastas;
- preparation of pasta samples of known composition and thermal history; method intercomparisons.

This report provides information on these method development and validation studies.

8. Project Summary

1. A range of pasta samples with known common wheat content and processing history have been prepared, and have been used for method development and validation studies, and for a methods intercomparison. This critical part of the project highlighted the requirement for 'standard' pastas, i.e. pasta samples with known composition and thermal history.
2. Capillary zone electrophoresis (CZE) was applied to the separation of common wheat D-genome gliadins but it did not show the anticipated analytical advantages. Separation times were long, day-to-day reproducibility was inconsistent, and correlation of CZE profiles to PAGE profiles was difficult.
3. An SDS-PAGE procedure has been developed which clearly separates common wheat ω -gliadins from other proteins extracted from pastas. The method uses 'mini-gels' and 10-15 samples can be examined simultaneously in a 45 minute electrophoretic run. It has been shown that other food ingredients (e.g. egg, meat, spinach) do not interfere with the assay. The target proteins are thermally stable therefore the method can be applied to pastas dried at elevated temperatures. Boiling of pasta samples and 'standards' prior to examination can be used to erase thermal history of pastas. This method shows advantage over the previously published A-PAGE procedure, and can be recommended for the detection of common wheat in Durum wheat pasta products. A recommended assay protocol is included in this report.
4. An HPLC procedure has been developed which provides greatly improved resolution of common wheat m-gliadins from other proteins extracted from pastas. The target peaks elute early in the chromatogram and are easily identified. An internal peak ratio procedure is used for quantitation. The target proteins are thermally stable and the method can be applied to pastas dried at elevated temperatures. Boiling of samples and 'standards' prior to examination can be used to erase thermal history of pastas. Food ingredients such as egg, meat, spinach, tomato, do not interfere with the assay. A recommended assay protocol is included in this report.
5. Monoclonal antibodies to D-genome gliadins have been produced and an ELISA has been developed which can be used to detect the presence of common wheat in Durum wheat pastas dried at elevated temperatures. Good discrimination between different levels of common wheat were obtained with reproducible calibration lines. A recommended assay protocol is included in this report.
6. Work has been undertaken to investigate the formation of Maillard Reaction products in pasta during production processing. This was performed to assess whether furosine and lysylpyrrolaldehyde could be

used as indicators of pasta thermal history. A number of model systems were examined and mathematical and graphical relationships are presented which describe MR product formation. It is proposed these relationships can be used to normalise results to account for differences in thermal history. Recommended assay protocols for the determination of furosine and lysylpyrrolaldehyde (MR products) are included in this report.

7. Work has been performed to assess protein extractability from pastas dried at different temperatures using dye-binding and Kjeldahl procedures. Information obtained suggests that these procedures can be used to classify pastas according to their thermal history and to normalise results to account for such differences. A recommended assay protocol is included in this report.
8. A polyclonal antibody against the common wheat protein Friabilin has been prepared and a sandwich ELISA developed using the polyclonal antibody as the capture antibody and the F7F monoclonal antibody for recognition. The developed assay is not susceptible to interferences from food ingredients, such as egg and spinach, which affected the ELISA designed on only the F7F monoclonal. Assay response is dependent upon the thermal history of the pasta examined, and this needs to be taken into account when calibrating the assay.
9. A revised assay format has been developed for screening pasta samples using a membrane strip assay and the F7F monoclonal antibody (against Friabilin) labelled with alkaline phosphatase for recognition. Coomassie Blue is used within the assay to characterise the thermal history of the pasta samples to be examined. The screening test is rapid (1 hour), only simple apparatus is required, 3% common wheat in pasta can be detected, and a permanent record of test results is obtained. A recommended assay protocol is included in this report.
10. A methods intercomparison has been held to evaluate developed methods of analysis. Samples of pasta were distributed to Partners numerically coded; Partners were requested to determine the common wheat content of these 'unknowns'. The results have shown that two or more methods of analysis, when used in concert, will provide reliable complementary evidence regarding the common wheat content of supposed Durum pasta samples.

9. Recommendations For Further Work

1. If results obtained using the developed methods are to be comparable between laboratories (and Countries) analysts require access to a range of Standard Pasta samples with known composition and thermal history. These samples would be used for assay calibration and for analytical

quality control. Such 'standards' do not currently exist; this should be addressed. More detail is provided in section 6 of this report.

2. A wider range of common and Durum wheats from different geographic origins (Turkey, Greece, France, UK, N America etc.) need to be examined.
3. The intercomparison study was of limited size (number of participants and range of samples examined), and a larger scale intercomparison exercise should be performed to more thoroughly assess the performance of the developed methods.
4. Methods have not been applied to the examination of the growing range of fresh and filled pastas in the marketplace. This needs to be addressed.
5. The immunoassay methods have been developed by the Partners but they will not be available to the analytical community if they are not commercialised. This needs to be addressed.
6. A one-day workshop should be held to disseminate details of the project and the developed methodologies to EU trade and enforcement bodies.

10. Dissemination Plans

1. Professor Griffin is exploring the commercialisation of the developed immunoassay with a UK diagnostics company. If this collaboration is successful the assay will be advertised internationally.
2. The revised HPLC procedure for ω -gliadins will be published in the scientific literature. This method is currently being used by the LGC for the examination of commercial pasta products in the UK.
3. The revised assay formats for the detection and determination of Friabilin will be marketed by Rhone Diagnostics Technologies Ltd internationally.
4. The SDS PAGE procedure will be published in the scientific literature and is currently used by INRA and the LGC for examination of commercial pasta samples.
5. The results of the project will be presented at scientific meetings by the Project Coordinator as the opportunities arise.
6. A paper will be published in the Scientific literature which reviews the overall actions and conclusions from the project.

7. Professor Resmini will publish the results of the studies on the products of the Maillard Reaction formed on pasta drying.
8. Results from the study will be disseminated via a workshop if the Commission can fund such an exercise.
9. Results of the project will be presented to the UK Ministry of Agriculture Working Party on Food Authenticity Methods Sub-Group.

Principles of methods

1. Electrophoresis and quantitation of 1D- ω -gliadin fractions

The research has focused on the ω -gliadin fraction that comprises the most heat-resistant proteins of the wheat kernel (because they are sulfur-free and therefore unable to form S-S bonded aggregates upon heating). After electrophoretic fractionation on a conventional polyacrylamide gel, the slow-moving triplet bands, that are specifically encoded by genes on the chromosome 1D of common wheat, may be used for common wheat determination. For that, these 1D- ω -gliadin bands may be either quantitated using a densitometer, or simply assessed by visual examination on the basis of a set of standard mixtures. In addition, to erase the possible differences in the level of denaturation due to various drying conditions (and also to remove any background in the pattern and to restrict the analysis to the only ω -gliadin fractions), all samples are boiled during 15 min before extracting the gliadins.

2) RP-HPLC and immunodetection of γ -gliadins

In this method, the detection of common wheat is based on another fraction of gliadins, which is identified through reversed-phase high-performance liquid chromatography (RP-HPLC) or an immunochemical method.

RP-HPLC of hexaploid and durum wheat gliadins showed that peaks eluting between 47-49 min represented a fraction belonging to the group of γ -gliadins, that was also genome-D-specific proteins. The total peak area calculated from the elution profile at 206 nm forms the basis of determining the common wheat content of pasta samples. Furthermore, antisera were prepared using γ -gliadin isolated from this 47-49 min peak on RP-HPLC, so that the detection of common wheat could be alternatively possible by immunochemistry.

3. Immunochemical detection of common wheat specific albumins (Mb 0.28)

Two albumins specific of common wheat belonging to the family of α -amylase inhibitors have been purified. Monoclonal antibodies have been raised against one of them referred to as Mb 0.28. Among these antibodies, some were found to react with the native form of the protein only whereas some others react with the denatured form only. Interestingly, one antibody could be selected that correspond to an epitope present on the native as well as on the denatured form of the protein. The latter has therefore the potential to recognise the specific albumin indiscriminately in its native or denatured state and to detect common wheat in pasta samples after any kind of heat treatment by a very simple and rapid ELISA immunoassay. In addition, albumin Mb 028 proved to have a very constant concentration among various common wheat varieties.

4. Determination of specific albumins ("A", "B", "C") of common wheat

Three albumin electrophoretic fractions ("A", "B", "C") were identified as specific of common wheat. Conversely, a durum wheat specific fraction ("D1") was observed. Although these albumin fractions are denatured upon pasta drying at elevated temperatures, they are still partially extractable when using efficient solvents containing dissociating agents. Because there is a similar denaturation rate of "A", "B", "C" and "D1" proteins under heat treatments, the ratio (A+B+C)/D1 can be used to determine the common wheat content in a pasta sample, even if relatively high temperatures are used in drying.

5. Durum Test Immunoassay on *Friabilin*

Monoclonal antibodies were raised against purified *Friabilin*, a 14.7K basic protein, encoded on chromosome 5D of hexaploid wheats, believed to be integral in the mechanism of hard/soft characteristic of wheat flour. A specific monoclonal antibody, coded as F7F, appeared to be specific of the S-S bridge C-terminal region of *Friabilin*. Durum wheats contain no *Friabilin*. Two tests were developed: a semi-quantitative dipstick test and an ELISA test.

Review of Methods Based on Initial Objectives Proposed

1. The effects of Drying Temperature on the Quantitation of Developed Methods

Any quantitative method developed must be independent of the drying temperature since the drying temperature of a suspected adulterated sample would not be known, furthermore drying methods may change with new manufacturing processes. If this is not possible then alternative techniques of either (i) estimating the thermal history of the pasta or (ii) erasing the thermal history of the pasta sample must be available. Methods were initially tested with pasta samples prepared by Barilla dried at the three different temperatures of 60°C, 78°C/92°C and 100°C.

The isoelectric focusing method prepared by P. Resmini was unable to be used with pastas dried at 100°C and hence was not developed any further after June 1991. The immunochemical method of A. Paraf / P. Violle directed against the albumin Mb 0.28 was capable of detecting common wheat adulteration at all drying temperatures but standard adulteration curves were not comparable between pastas dried at different temperatures. This same criticism applied to all other methods *i.e.* electrophoresis of ω -gliadins, HPLC of γ -gliadins, and the immunochemical detection of friabilin. In general the sensitivity of each method decreased as the drying temperature increased. The most sensitive methods proved to be the immunochemical methods based on the albumins Mb 0.28 and friabilin, and both were capable of detecting common wheat adulteration of 3% (m/m) in pastas dried at 100°C.

In the electrophoretic method with ω -gliadins, attempts were made to overcome the problems of temperature on quantitation by erasing the thermal history of the pastas by boiling the sample for 15 min prior to assay. This proved to be only partially

successful since in 100°C dried pastas background absorbance (mistaken for ω -1D material when using densitometric measurements) made quantitation difficult.

Similarly, in the RP-HPLC method of γ -gliadins, the total peak area of a chromatogram extracted from a given weight of pasta gave some indication of the thermal history of the pasta since drying temperature was found to be directly related to the total peak area of the chromatogram.

RP-HPLC detection of ω -gliadins was also found to be possible in heated pastas. Results with ω -gliadins were found to be comparable to the γ -gliadin data. A major advantage of using RP-HPLC is that it can use both ω - and γ -gliadins as a method of common wheat detection.

2. Specificity and Genetic Variation

In June 1991 it was found that the specificity of the monoclonal antibodies recognising the common wheat albumin Mb 0.28 was not confined to common wheat varieties since some "false positives" were observed with some durum wheats. For instance, Barilla samples containing certain Italian durum wheat varieties were found to cross react with the monoclonal antibody raised to the Mb 0.28 protein. Although it may be possible to find other monoclonals raised against Mb 0.28 (*e.g.*, against the denatured form of the Mb 0.28) which do not react with durum varieties, the suitability of Mb 0.28 as a common wheat specific target protein came into question.

A comparable problem may also arise for the immunochemical detection method based on friabilin. The content of friabilin in most common wheats is fairly constant. However, it is known that some varieties of wheat do contain much lower levels of this starch binding protein, while recent studies on lipid-binding proteins (Marion, INRA-Nantes, France) tend to indicate that some spring varieties of common wheat lack one of the three friabilin electrophoretic components.

Similarly, the content of ω -gliadins and the common wheat specific γ -gliadin used in the RP-HPLC method varies with common wheat varieties making quantitation by these methods less accurate than would be desirable.

The immunochemical method based on the γ -gliadins (M. Griffin) has still not been fully tested. Antibodies raised to γ -gliadins extracted from 100°C dried pastas show considerable promise. The polyclonal antisera following affinity purification against durum gliadins cross react with all Barilla samples dried at 60°C, 78/92°C and 100°C and the antibody is capable of detecting common wheat at levels of 3% (m/m) in 100°C dried pastas. The method in its present form does however, suffer from the same problems as the RP-HPLC method *i.e.* these is an inverse relationship between drying temperature and sensitivity and the problem of genetic variation in common wheats.

3. Inter Laboratory Trials

Two inter laboratory trials were performed with three of the methods outlined - the electrophoresis of ω -gliadins, the RP-HPLC of γ -gliadins and the immunochemical detection of friabilin.

a) The first trial involved the interchanging of Barilla samples which had been used to construct the standard curves in each of the methods. The results from this trial indicated that, in the range 3% - 5% -12% - 15% and 25% common wheat content, all methods were reproducible and accurate when unknown samples were made from the same wheat as that used to construct the standard curves. Therefore the varietal variation could not be evaluated.

b) In 1992, because of an increasing need for a precise method for the determination of common wheat in the 0-10% range, a second trial was decided, including unknown pastas containing small percentages of common wheat and dried at 92+80°C or 100°C.

A first batch of 10 unknown samples (labelled 1-10) was therefore sent by Dr. Landi to all participants in April 1992 and a second batch consisting of 3 samples (labelled R, X and Y) was sent in September 1992.

The three above-mentioned methods have been carried out on these samples. In the case of the Rhône-Poulenc Elisa Kit, the analyses have been carried out by Rhône-Poulenc and independently by Dr. I. Lumley (Laboratory of Government Chemists, Middlesex, UK). The results (% of adulteration by common wheat) of the four analyses are given in **Table I**.

The results of this second trial showed considerable differences between the methods. The least accurate measurements appeared to be obtained with unknown samples dried at 110°C.

- Although indicating a reliable picture of the general trend of results, the electrophoretic method using ω -gliadins tended to underestimate the amount of common wheat adulteration, confirming therefore its semi-quantitative characteristic.
- The RP-HPLC method although accurate in eight out of ten samples gave two inaccurate results (samples 1 and 4).
- The immunochemical method involving friabilin was the most accurate method in the hands of the proposer (Rhône-Poulenc laboratories). All the results differed by less than 1% from the reference values, except sample 7 which was slightly underestimated. In contrast, when this analysis was carried out at LGC, more discrepancies were observed, although there was still a reliable approach of the general trend of results.

4. Comparison of Methods Carried out at the Laboratory of Government Chemists

At the last meeting of the BCR Pasta Working Group, it was agreed that the Laboratory of Government Chemists (Dr. I. Lumley) should estimate the amount of common wheat in 10 industrially prepared pasta samples using the three analytical procedures under consideration by the Group. This was carried out in January 1994.

a) Sample Information

Ten samples received randomly numbered 1 to 10 were used. They consisted of dried pasta manufactured by Barilla, containing varying proportions of common wheat, dried at either 92/80°C or 100°C

b) Sample Preparation

Each sample was ground with a food blender and stored in a plastic jar at room temperature.

c) Methods of Analysis

The analytical procedures followed were:

- i) Electrophoresis of ω -gliadins, based on the method of Autran.
- ii) RP-HPLC of γ - and ω -gliadins, based on the method of Griffin and Barnwell.
- iii) ELISA (enzyme linked immunosorbent assay) of the protein friabilin, marketed by Rhone Poulenc Diagnostics.

Triplicate results were obtained for each sample by each method; calibration was against standards prepared by Barilla for the Working Group.

Analysts were not informed of the composition of the samples submitted for analysis; results were obtained 'blind'.

d) Analytical Results

Results are shown in **Table II**.

e) Additional Information

i) Electrophoresis of ω -gliadins

The three results reported were obtained from separate sample extracts and were electrophoresed on separate days. The drying temperature of the unknown pastas was estimated from the electrophoretic profile density and the corresponding Barilla 'standards' used for assay calibration. Fresh standards were prepared for each assay and electrophoresed on the same gel as sample extracts.

Eight and ten replicate loadings of an extract from sample 7 were electrophoresed on 2 gels to assess the repeatability of peak height rates measurement by laser densitometry. The coefficient of variation was found to be $\pm 7\%$.

ii) RP-HPLC of γ -gliadins

The three results reported were obtained from separate sample extracts and chromatographed on separate days. The drying temperature of the 'unknown pastas' was estimated from the total chromatographic peak area and the corresponding Barilla 'standards' used for assay calibration.

Precipitation of proteins in some sample extracts was experienced which caused analytical difficulties. [However, precipitation could be a problem only if the extraction procedure is not adhered to or if the samples are stored for a long period of time *i.e.* 10 hours or more on an autosampler]. It can also be seen that this method produced a 'zero' result for some samples which contained common

wheat, *i.e.* samples 5, 7 and 10. This might be due to protein precipitation and loss of chromatographic resolution of the small gliadin peak of interest. This requires further investigation.

iii) Immunoassay of Friabilin

Each ELISA result reported is the mean of 4 results obtained from 4 aliquots of each sample applied to the microtitre plate. The pasta drying temperature was estimated from the electrophoretic and HPLC profiles and the corresponding Barilla 'standards' used for assay calibration.

Conclusions

This final report is a true reflection of all our group's efforts to develop new methods of determination of common wheat in pasta products that are compatible with the use of elevated temperatures in the pasta industries.

Three methods seem to be "workable", as indicated by the last comparison. On the basis of the results shown in Table II (average of triplicate results), *electrophoresis of ω -gliadin* gave quite encouraging results with no major error compared with the actual values. *RP-HPLC of γ -gliadins* gave a reliable approach of the general trend of results with some underestimation of the amount of common wheat for some pasta samples dried at 100°C. *Immunoassay of friabilin* was the most accurate at low additions of common wheat but showed some significantly biased results at high concentrations.

However, if considering samples with totally unknown drying conditions and variety composition, *there is presently no one method that can be proposed as a reference method for the European Union*. Each method suffers from its own problems likely to cause inaccuracies if quantitation of common wheat adulteration is required as summarised hereunder.

	Specificity/ common wheat	Indep./ variety	Indep./ 92°C drying θ	Indep./ 100°C drying θ	Accuracy at very low % adulteration	State of the protocol
	-----	-----	-----	-----	-----	-----
Electrophoresis ω -gliadin	+++	+	+++	++	+ / +++	+++
HPLC γ -gliadin	+++	+	+++	++	+ / +++	+++
Immuno/albumin 0.28	+	++	++	+	+++	++
IEF albumins	+++	+++	++	-	++	+
Immuno/ <i>Friabilin</i>	+++	+ / +++	+++	++	+++	+++

The present methods *if used in combination with one another* may be acceptable since this would alleviate some of the problems associated with the thermal history of the sample and/or genetic variation. On the other hand, even in the future, two methods based on two genetically different protein markers should certainly be retained.

However, the present methods can only be recommended for pasta samples dried at the temperatures already tested, and with pasta not containing any other additives *e.g.*

egg protein, soya protein, milk protein or spinach. Whereas the immunochemical method on albumin Mb 0.28 is likely to be less sensitive to competition by other proteins (because it is based on a sandwich ELISA), the immunoassay on friabilin is known to suffer problems when egg albumin is present in the pasta sample. The effect of water soluble additives on RP-HPLC and electrophoresis is likely to be negligible since these methods are based on alcohol soluble proteins. However, this is yet to be tested in depth.

The final recommendation would be to that *further research and developmental work is required* in order to achieve the original objectives of the project.

Table I. Results (% adulteration) of the three methods on 1992 inter laboratory trials

1) Samples received in April 1992 (drying temperature: 92+80°C (1) or 100°C (2))

Method:	<i>Actual % of common wheat (a)</i>	A-PAGE ω -gliadin (b)	RP-HPLC γ -gliadins (c)	Rhône-Poulenc Elisa Kit Rhône Poulenc (d)	Elisa Kit LGC (e)
Sample Number:					
1 (2)	3-4	1-2	6.2	2-3	2
2 (1)	1-2	0-1	2.5	1-2	3
3 (1)	9-10	4-5	8.4	7-8	13
4 (2)	0-1	2-3	4.0	0	0
5 (2)	4-5	1-3	6.6	4	2
6 (1)	6-7	3-4	6.1	7	10
7 (2)	9-10	3-4	7.8	6-7	4
8 (2)	1-2	2-3	< 3.0	1	1
9 (1)	0-1	0-1	0	0	1
10 (1)	4-5	1-2	4.8	5-6	7

2) Samples received in September 1992 (drying temperature: 110°C)

Method:	<i>Actual % of common wheat (a)</i>	A-PAGE ω -gliadin (b)	RP-HPLC γ -gliadins (c)	Rhône-Poulenc Elisa Kit Rhône Poulenc (d)	Elisa Kit LGC (e)
Sample Number:					
R	0	2	0	0	-
X	1.5	1-2	3	1-2	-
Y	4.5	2-3	4-6	3-4	-

(a) Results obtained from Dr. Landi (Barilla, Italy)

- (b) Electrophoresis of ω -gliadins: Laboratoire de Technologie des Céréales INRA, Montpellier, France (Dr. J.C. Autran)
- (c) RP-HPLC of gliadins: The Nottingham Trent University, UK (Prof. M. Griffin)
- (d) Rhône-Poulenc Diagnostics Elisa Kit : analyses carried out by Rhône-Poulenc, Glasgow, UK (Prof. W. Stimson and Mr. M. Bony)
- (e) Rhône-Poulenc Diagnostics Elisa Kit : analyses carried out independently by Laboratory of Government Chemists, Middlesex, UK (Dr. I. Lumley)

Table II. Analytical results of the three methods (comparison carried out in January 1994 at the Laboratory of Government Chemists by Dr. I. Lumley)

i) Electrophoresis of ω -gliadins

Barilla No. %	Results Obtained %	Average value %	Range %	Actual value °C	Drying Temp.
1	6, 5, 3	5	3-6	3-4	100
2	2, 1, 1	1	1-2	1-2	92 + 80
3	10, 12, 9	10	9-12	9-10	92 + 80
4	0, 0, 0	0	0	0-1	100
5	0, 4, 4	3	0-4	4-5	100
6	7, 6, 7	7	6-7	6-7	92 + 80
7	11, 8, 4	8	4-11	9-10	100
8	3, 3, 0	2	0-3	1-2	100
9	1, 3, 0	1	0-3	0-1	92 + 80
10	3, 5, 8	5	3-8	4-5	92 + 80

ii) RP-HPLC of γ -gliadins

Barilla No. %	Results Obtained %	Average value %	Range %	Actual value °C	Drying Temp.
1	3, 3, 0	2	0-3	3-4	100
2	0, 0, 0	0	0	1-2	92 + 80
3	11, 12, 12	12	11-12	9-10	92 + 80
4	0, 0, 0	0	0	0-1	100
5	5, 4, 0	3	0-5	4-5	100
6	7, 8, 8	8	7-8	6-7	92 + 80
7	7, 4, 0	4	0-7	9-10	100
8	0, 0, 0	0	0	1-2	100
9	0, 0, 0	0	0	0-1	92 + 80
10	5, 4, 0	3	0-5	4-5	92 + 80

iii) Immunoassay of Friabilin

Barilla No. %	Results Obtained %	Average value %	Range %	Actual value °C	Drying Temp.
1	5, 3, 4	4	3-5	3-4	100
2	3, 2, 0	2	0-3	1-2	92 + 80
3	16, 13, 16	15	13-16	9-10	92 + 80
4	1, 0, 1	1	0-1	0-1	100
5	7, 3, 5	5	3-7	4-5	100

6	12, 11, 12	12	11-12	6-7	92 + 80
7	16, 10, 10	12	10-16	9-10	100
8	3, 3, 1	2	1-3	1-2	100
9	1, 0, 4	2	0-4	0-1	92 + 80
10	9, 10, 9	9	9-10	4-5	92 + 80

Annex I

Protocol of Determination of Common Wheat in Durum Wheat Pasta Submitted to High-Temperature Drying, by Polyacrylamide Gel Electrophoresis of ω -gliadins

J.-C. Autran and J. Bonicel

**Laboratoire de Technologie des Céréales
INRA, 2 Place Viala, 34060 Montpellier Cedex 1, France**

1. PRINCIPLE

Common wheat (*Triticum aestivum* L.) and durum wheat (*Triticum durum* Desf.) belong to two different cereal species. The first contains three genomes (A, B and D), and the second only two (A and B). For this reason, some proteins encoded by genes on genome D are specific for common wheat. Among them, certain albumin (water-soluble), peroxidase and gliadin (ethanol-soluble) have been identified during the last thirty years.

Both the Italian (2, 8, 9, 10) and French (5, 6) methods of common wheat content determination have been based upon these differences in protein composition: the amount of specific proteins could be determined by densitometric scanning or visual examination of electrophoretic patterns (after protein staining, or peroxidase assay). The main limitation of these two methods, however, came from protein denaturation during high temperature processes (4, 6) which have been increasingly used by pasta industries during these last ten years (3). In contrast, certain slow-moving ethanol soluble proteins (ω -gliadins) are both specific for common wheat and heat resistant (1, 3, 7).

The purpose of this method is to determine the common wheat (*Triticum aestivum*) content in durum wheat (*Triticum durum*) products (pasta, couscous semolina) including high-temperature dried or pre-cooked products. This document describes a method whereby genome D - specific gliadin proteins are extracted from pasta, separated in 1.5 mm thick PAGE slab gels containing a pH 3.1 aluminium lactate buffer and quantified by densitometric scanning (in comparison with a set of standard common wheat / durum wheat mixtures) to determine the content in common wheat.

To erase any difference in the "thermal history" of the samples, which may have led to different levels of denaturation, both unknown pasta samples and standard mixtures are boiled 15 min, freeze-dried and ground previous to the gliadin extraction step.

2. APPARATUS AND SUPPLIES

2.1 Vertical Electrophoresis Unit

LKB Model 2001 with Teflon slot formers for 15 sample wells (1.5 x 7.0 mm).

2.2 Stabilized Power Supply

Numerous commercial D.C. power supplies which provide stable and constant voltages up to 1000 volts and constant current up to 300 mA are suitable.

- (i) Pharmacia-LKB Models 2301 or 2197, or
- (ii) ISCO Model 494, or
- (iii) Bio-Rad Model 1000/500

2.3 Densitometer

Pharmacia-LKB laser densitometer, connected to a PC-compatible computer system equipped with a software for data processing, integration and storage. Numerous systems are suitable.

- (i) Spectra Physics Station
- (ii) Kontron
- (iii) Beckman Gold
- (iv) Waters

2.4 Thermostated Refrigeration Circulating Bath

A circulating fluid cooling bath to maintain the electrophoresis unit at 14°C.

- (i) Forma Scientific Model 2095, or (
- ii) Haake Model DI G, or
- (iii) Pharmacia-LKB Multi Temp II

2.5 Micro Centrifuge

A tabletop micro centrifuge which can provide a minimum centrifuge force of 10,000 g for clarifying protein solutions, Eppendorf Model 5414, equipped with Eppendorf tubes, 2 ml, no 0030.120.094

2.6 Magnetic Stirrer

Teflon-coated magnetic stirrer bars, 2.5 and 5.0 cm long.

2.7 Tube Mixer

Tube mixer, type IKA Vibrax V x R

2.8 Gel Shaker

Type Bioblock Scientific

2.9 Micro pipette

Hand-held repeater pipetter with 2.5 ml disposable tips to deliver volumes of 50-250 μ l.

2.10 Syringe

Micro syringe, 25 μ l capacity, equipped with stainless steel plungers. Hamilton Gas-Tight Model 1702.

2.11 Plastic Storage Containers

Rigid polyethylene containers approximately 30 x 17 x 5 cm, for gel staining and storage.

2.12 Laboratory Freeze-Drier

- (i) Virtis Freezemobile 12, or
- (ii) Serail RP 2 V

2.13 Drying Chamber

Type SECASI

2.14 Sheeter

Type De Lellis

2.15 Sample Grinder

Grinder, type MIAG, Braunschweig, AZ 53114

2.16 Cooking Facilities

Heating plate, 2.5 l pans, tea infuser.

2.17 Laboratory Refrigerator or Cold Room

Laboratory refrigerator, equipped with a freezer and thermostated sections for storage of solutions, chemicals and samples

2.18 Analytical Balance

A balance sensitive to ± 0.1 mg for weighing chemicals and samples

2.19 pH Meter

A pH meter with an accuracy of ± 0.05 pH units

2.20 Moisture Meter

2.21 Water Purification System

A deionization system to provide high quality water having resistance $> > 10$ megaohms, *e.g.* Milli Q water purification system.

3. CHEMICALS

Acrylamide (electrophoresis grade): Eastman Kodak, LKB, or Biorad

Aluminium lactate (Fluka, lactic acid, aluminium salt)

Ascorbic acid

Coomassie Brilliant Blue R250

2-Chloro-ethanol

Crystallised Violet

Ethanol

Ferrous sulphate heptahydrate

Hydrogen peroxide (30%)

Lactic acid (density 1.24)

N,N'-Methylene-*bis*-acrylamide (electrophoresis grade): Eastman Kodak, LKB, or Biorad

Sodium chloride

Sucrose

Trichloroacetic acid

Water (deionized)

4. WHEAT SAMPLES

Pure durum wheat semolina representative of the main durum wheat cultivars.

Common wheat flour representative of the main common wheat cultivars.

5. SOLUTIONS

5.1 Extraction Solution

2-Chloro-ethanol	25 ml
Crystallised violet	20 mg
Sucrose	30 g
Dissolve and make up to 100 ml with deionized water	
Store at room temperature	

5.2 Aluminium Lactate Stock Solution

Aluminium lactate	50 g
Lactic acid	55 ml
Dissolve and make up to 1 l with deionized water	
Store at 4°C	

5.3 Buffer Solution

Aluminium lactate stock solution (5.2)	250 ml
Make up to 5 l with deionized water	
Measure pH which should be 3.1.	

5.4 Gel Solution

WARNING! Acrylamide monomer is a neurotoxic substance which can be absorbed through the skin. Caution should be taken when handling both the crystalline powder and gel solution.

Acrylamide	80.0 g
N,N'-Methylene- <i>bis</i> -acrylamide	4.0 g
Ascorbic acid	1.0 g
Ferrous sulphate heptahydrate	0.033 g
Aluminium lactate stock solution (5.2)	250.0 ml
Dissolve and make up to 1 l with deionized water	

5.5 Catalyst Solution

Hydrogen peroxide (30%)	0.33 ml
Dissolve and make up to 10 ml with deionized water	
Store at 4°C	

Renew every day

5.6 Trichloroacetic Acid Solution

WARNING! Trichloroacetic acid is corrosive

Trichloroacetic Acid 120.0 g
Dissolve and make up to 1 l with water

5.7 Coomassie Brilliant Blue R250 (CCB) Stock Solution

Dissolve 5.0 g CCB by stirring in 1 l of 95% ethanol for 30 min

5.8 Staining Solution

Add 5.0 ml of CBB solution (5.7) to 95 ml trichloroacetic acid solution (5.6).
Prepare shortly before use

6. SAMPLE PREPARATION

6.1 Preparation of the set of standard mixtures

Prepare a series of mixtures of durum wheat semolinas with different percentages of common wheat flour, *i.e.* 0, 3, 6, 9, 12, 15, 20, 40, 60, and 100%.

Use 50 g amounts of each mixture. Determine the moisture content using the moisture meter and add the amount of water required so as the water content of the dough is 38 %. Prepare homogeneous doughs by a 1-min hand kneading. Sheet the doughs through the sheeter so as to obtain a 1 mm thick sheeted dough.

Dry the series of sheeted doughs in the drying chamber (2.13) so as to reduce the water content to 13% in 5 hrs. A typical drying diagram may be the following:

- 1 hr of linear increase of the temperature from 35 to 90°C with a constant 88% relative humidity,
- 2 hrs at constant 90°C temperature with a linear decrease of the relative humidity from 88 to 77%,
- 2 hrs of linear decrease of the temperature from 90 to 40°C with a linear decrease of the relative humidity from 77 to 70%.

6.2 Heat Treatment of Sheeted Doughs and Unknown Pasta Samples

Cook each 50 g sheeted pasta sample or unknown pasta sample for 15 min in 1.5 l of boiling natural spring water to which 10.6 g of NaCl was added. After cooking, allow

pasta to drain off and cool. Then freeze-dry it. Grind the freeze-dried products using the sample grinder (2.15) and homogenise the powder.

6.3 Heat Treatment of Semolina or Couscous

Put 10 g of semolina or couscous in the tea infuser. Cook for 15 min in 1.5 l of boiling natural spring water to which 10.6 g of NaCl was added. After cooking, allow the products to drain off and cool. Then freeze-dry them. Grind the products using the sample grinder and homogenise the powder.

7. PROCEDURE

7.1 Protein extraction

Weigh 50 mg of each thermally-treated ground pasta (6.2), semolina, or couscous sample (6.3) into a centrifuge tube (2.5) and add 400 μ l extraction solution (5.1). Mix contents with Vibrax mixer for 1 h at room temperature. Centrifuge at 14,000 rpm for 10 min. Use the supernatant for sample loading (7.3) in the electrophoretic gel.

7.2 Gel preparation

Assemble gel cassettes (2.1) with 1.5 mm thick spacers, as described in the manufacturer's instructions.

Store at 4°C in the refrigerator or cold room (2.17).

To prepare each gel, combine 40 ml of gel solution (5.4) and 50 μ l of catalyst solution (5.5). Swirl the solution gently by hand for 10 s, taking care not to incorporate air bubbles. Quickly pour the solution into the cassette in a steady stream to minimise the introduction of air. Immediately, once the cassette is filled, insert the 15-well slot former. Polymerization is complete in 5-10 min at 4°C.

7.3 Sample loading

Carefully remove the slot former and fill the sample wells with buffer solution (5.3) using a micro pipette (2.9) to prevent dehydration of the gel. Using a micro syringe (2.10), deposit 6 μ l of sample extract (7.1) in the bottom of the wells. This step is made easier due to the crystallised violet in the stock solution. (The dense sample extract underlayers the buffer solution in the wells). Each gel must contain both unknown samples and the standard set.

Because the analysis of unknown samples is made with reference to standards containing a known quantity of common wheat, the set of standards must be incorporated in the same electrophoretic gel in which the unknown samples are studied (**Fig. 1**).

7.4 Assembly of the Electrophoresis Unit

Attach the upper buffer chamber to the tops of the gel cassettes. Assemble the components of the lower chamber according to manufacturer's instructions. Place the upper chamber / gel cassette assembly into the lower chamber which has been filled with 3.6 l of buffer solution (5.3). Fill the upper chamber with buffer solution (5.3), place the safety lid onto the unit and connect the electrodes to the power supply (2.2). Ensure that the upper chamber electrode is the anode and that it is connected to the positive (+) pole of the power supply, while the lower chamber electrode is the cathode and is connected to the negative (—) pole of the power supply.

7.5 Electrophoresis

Set the circulating water bath (2.4) to maintain the electrophoresis unit at 14°C during the run. Use a constant voltage of 500 V during the run, which corresponds to an average intensity of 60 mA. Allow the run to proceed for 120 min. Then turn off power supply, disconnect safety lid, remove upper chamber assembly and pour off upper buffer.

WARNING! The high voltages are potentially lethal. Ensure that the electrophoresis apparatus is used safely according to the manufacturer's instructions and no electric leaks are present.

7.6 Staining

Detach the gel cassettes from the electrophoresis unit. Open cassettes, remove gels and place each in a plastic container (2.11) containing 100 ml of staining solution (5.8). Place covered containers on the shaker (2.8) to agitate contents gently at approximately 60 rpm for 4-18 h. Destaining is not required. However, the precipitated stain must be removed by washing the gels in water before the densitometric or photographic steps.

8. DENSITOMETRIC SCANNING

Place the cleaned gel on the transilluminator of the densitometer (2.3) and position it. Scan the gels using the following settings: 200 mm/min (scan speed), 100 mm (scanning length), 1.0 optical density. After scanning each pattern, the curves are processed by the computer software.

9. PATTERN INTERPRETATION

Identify the peaks corresponding to the D-genome-specific (slow-moving) ω -gliadin bands on the densitometric curves. For each pattern (standards and unknown samples),

calculate the total area under the densitometric tracing of the D-genome ω -gliadin peaks. Plot these data against the known common wheat percentages (**Fig. 2**).

10. EXPRESSION OF RESULTS

The common wheat content, expressed in gram per cent of the product, is estimated on the basis of the calibration curve (see 9.).

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semolina and in macaroni products (in Italian). *Tecnica Molitoria*, 27 (10), 97-109.

Annex II

RP-HPLC Detection and Quantification of Common Wheat Adulteration of Durum Wheat Pasta Dried at High Temperature

M. Griffin and P. Barnwell

**Dept of Life Sciences, Nottingham Polytechnic
Nottingham NG11 8NS. U.K**

PRINCIPLE

This technique detects the presence of common wheat gliadins in pasta samples dried at high temperatures using reversed phase - high performance liquid chromatography.

APPARATUS AND SUPPLIES

Super Junior S coffee grinder, Moulinex, France

Polyethylene vials (20 cm³)

Flask shaker, Gallenkamp, UK

MSE Micro Centaur microfuge, Fisons Scientific Equipment, UK

Microfuge tubes

Plastipak syringes (5 cm³)

Swinnex syringe filters, Millipore, UK

0.45 μ m filters, Millipore

Jouan RC 10.22 centrifugal evaporator, France

HPLC system: Waters Associates model 6000A and Waters Millipore 510 solvent delivery systems, Waters Millipore 680 automated gradient controller, LKB 2157

autosampler, LKB 2238 Uvicord SII single beam UV detector, Nelson Analytical software, Tecam C-100 water circulator, Waters Delta Pak C18 15 μ m particle size

(300 x 4 mm) column, nucleosil based C18 cartridge guard column (30 x 4 mm)

Autosampler vials, screw caps and Teflon discs, Chromatography Supplies, UK.

CHEMICALS

DL- dithiothreitol, Sigma, UK

Ethanol absolute, SpectrosoL BDH, UK

Acetonitrile, HiPerSolv, BDH

Trifluoroacetic acid, SpectrosoL, BDH.

SOLUTIONS

Extracting medium:	70% (v/v) aqueous ethanol + 1% (w/v) dithiothreitol
Resuspending medium:	100% (v/v) ethanol + 1% (w/v) dithiothreitol
HPLC solvent A:	15% (v/v) aqueous acetonitrile + 0.1% (v/v) trifluoroacetic acid
HPLC solvent B:	80% (v/v) aqueous acetonitrile + 0.1% trifluoroacetic acid

PROCEDURE

Protein extraction

One g ground pasta samples were mixed with 4 cm³ extracting medium and gliadins extracted at room temperature with vigorous shaking (Gallenkamp shaker setting 10) overnight.

Extracted solutions were clarified by centrifugation (13000 g x 10 min) and filtered through a 0.45 μ m pore size filter. Following filtration solutions were reduced to 30% volume in a centrifugal evaporator (Jouan evaporator setting 2, 60 min), then taken back up to 50% volume with resuspending medium.

RP-HPLC separation

All chromatographic equipment used in the separation of extracted gliadins is listed under Apparatus and Supplies. 100 μ l samples were injected onto a Waters Delta Pak C18 column (15 μ m particle size) maintained at 40°C. A linear 45 min gradient from solvents A:B, ratio of 77:23 to 50:50 by volume with a hold at final concentrations for 10 min and a flow rate of 1.0 cm³ min⁻¹. Eluted proteins were monitored at 206 nm.

An example elution profile for pastas dried at 60°C, 92°C + 78°C and 100°C (Barilla samples) adulterated with 15% common wheat flour is shown in **Fig. 1, 2 and 3**. **Fig. 4** shows combined elution profiles.

Standard curves showing peak area of normalised adulterant peak (47 min) plotted against percentage adulteration of common wheat (Barilla samples) is shown in **Fig. 5, 6 and 7** for pasta samples dried at 60°C, 92 + 78°C and 100°C and combined standard curves are presented in **Fig. 8**.

Annex IIIa

Immunochemical detection of common wheat specific albumins

P. Violle and A. Paraf

Laboratoire d'Immunologie, INRA, Centre de Recherches de Tours-Nouzilly,
Domaine de l'Orfrasière-Nouzilly, 37380 Monnaie, France

Our aim was to identify by an immunological test the quantitative adulteration of pasta made of durum wheat by common wheat. The choice of the target protein was driven as following:

- a) Protein from the D genome
- b) Protein water-soluble
- c) Protein present with the same ratio in all varieties of common wheat

Thus, albumins Mb 0.28 and Mb 0.19 were the chosen proteins.

PROCEDURE

- 1) Purify these two proteins.
- 2) Make polyclonal and monoclonal antibodies against these proteins.
- 3) Design an extraction procedure from pasta, whatever technology was applied (temperature, humidity, drying process, etc.).
- 4) Design the immunological format to quantitative common wheat present in pasta.

1) Purification

Proteins Mb 0.28 and Mb 0.19 from common wheat cultivar Camp Remy were water extracted and lyophilised. Then, after rehydration, the following steps were:

- a) $\text{SO}_4(\text{NH}_4)_2$ precipitation,
- b) G 75 chromatography
- c) Q Sepharose chromatography for Mb 0.19 and FPLC mono Q chromatography for Mb 0.28.

2) Polyclonal antibodies

Purified antigens induced polyclonal antibodies in mice, rabbits and goats. After exhaustion of cross reactive antibodies on column of durum wheat, specific antibodies for common wheat were obtained. On **Fig. 1** an example is given for 2 mice sera immunised with native or denatured Mb 0.28.

3) Monoclonal antibodies

A Balb/C mouse immunised 4 times with the native Mb 0.28 protein gave 18 monoclonal antibodies which were studied by either indirect ELISA (**Table I**) or sandwich ELISA (**Table II**).

From these tables we can conclude:

- a) All mAbs from the TN group are specific of the 0.28 protein, native or heat denatured (by indirect ELISA) while being essentially specific of the native form by sandwich ELISA. In every instance flour from common wheat was identified while flour from durum wheat was not recognized.
- b) Most mAbs of the TD group resemble to those of the TN group but there was a slight cross reaction with the durum wheat and epitopes identified were more thermoresistant than those of the TN group.
- c) Most mAbs of the D group not only bound Mb 0.28 native or heat denatured, but also Mb 0.19, and also strongly to durum wheat when tested by indirect ELISA. However when tested by sandwich ELISA two (D5 and D6) did not bound to durum wheat.

4) Design of an extraction procedure from pasta

The ground pasta was dissolved in an alkaline solution with a reducing agent for 5 hrs. Sandwich ELISA was performed by whatever monoclonal antibody bound to plastic while, after antigen has been captured, goat polyclonal antibodies were used for identification and quantification of common wheat in pasta. On **Fig. 2** are presented results with pasta prepared by a drying process either at 55°C or at 100°C for two hours (a very strong treatment not used in industry). It can be concluded that despite an intensive treatment it is possible to detect common wheat at low concentration (< 3%). But quantification is not possible when pasta has received an unknown treatment.

5) Effect of additives and wheat varieties

Thanks to the sandwich ELISA capturing antigen with the monoclonal antibody, the assay is totally independent of additives. Adulteration is perfectly detected and quantified in pastas containing egg, milk or soya flour (**Fig. 3**).

On 40 different varieties of common wheat, concentration of Mb 0.28 tested by sandwich ELISA was about the same. For instance, for pastas containing 20% common wheat adulteration, optical density varied from 1.076 to 1.262. Nevertheless, a few durum wheat varieties presented a low but non nil reactivity. Pastas produced with those varieties was detected as false positive samples (**Table III**).

Consequently, we should now focus our efforts in two different directions which are complementary:

- a) To look for monoclonal antibodies unable to detect any durum wheat and specific of thermoresistant epitopes. To raise monoclonal antibodies against denatured Mb 0.28 could be one solution to investigate this way.
- b) To modify the extraction procedure so that denatured Mb 0.28 protein can be equally isolated whatever was the technology applied to pasta.

CONCLUSIONS

- 1) Mb 0.28, genetically specific to common wheat, seems to be a good target protein which showed practically the same concentration in 40 different varieties of common wheat used in France.
- 2) Eighteen different monoclonal antibodies, raised against native Mb 0.28, have been isolated. A few durum wheat varieties showed a low reactivity on the test with these antibodies.
- 3) When technologies applied to pasta were known, these mAbs allowed accurate determination of common wheat. However, when pasta was of unknown origin, the test was not quantitative.
- 4) We should now select mAbs specific of thermoresistant epitopes and unable to detect any durum wheat.
- 5) A modified extraction procedure should be designed to annul the effect of temperature and humidity in pasta during the drying process.

Compared with other methods, the test showed the following advantages:

- The immunological format tests are perfectly adapted to develop handy kits needed by the industry.
- The sandwich ELISA assay capturing the antigen from the mixture fit with pasta samples containing any additives such as egg, milk, or soya. [If a defrauder knows an additive able to mask his adulteration, he will certainly use it].

At present, this assay is not working because of the false positive obtained with several durum wheat varieties. However, this major defect does not affect its high potentialities. Because ways exist to solve its specific troubles, this test should not be abandoned and new investigations should be developed, especially if the other methods did not give their expected results.

Annex IIIb

Protocol of Immunochemical Detection and Quantification of Common Wheat in Pasta Dried at High Temperature

P. Violle⁽¹⁾ and A. Paraf⁽²⁾

(1) LTC-INRA, 2 place Viala, 34060 Montpellier Cedex 01, France

(2) Laboratoire d'Immunologie, INRA, Centre de Recherches de Tours-Nouzilly, 37380 Monnaie, France

1. PRINCIPLE

The test detects the presence of a heat resistant epitope of a specific albumin of common wheat. This protein is revealed by a sandwich ELISA Test: a mAb captures the protein. A polyclonal serum is used to reveal it.

2. APPARATUS AND SUPPLIES

Grinder KT30, Falling Number, Sweden

Extracting tubes: Polypropylene 17x100 mm (15 ml)

Rotary mixer: Reax2 Heidolph (Germany)

ELISA plates Nunc Maxisorb 96 wells

Nunc Immuno wash 12

Plates reader: Titertek Multiskan MCC, Flow Lab Ltd, UK

3. CHEMICALS

ABTS 2,2'-Azino-di(3-ethylbenzothiazolinesulfonate (6)), Boehringer

Citric acid

Caster non-fat-milk

Di-sodium hydrogen phosphate

Di-sodium carbonate

Glycine

Potassium chloride

Potassium dihydrogen phosphate

Sodium chloride

Sodium dodecyl sulfate

Sodium hydrogen carbonate

Tween 20

2-Mercaptoethanol

Antibodies

TD1 Monoclonal antibody against native Mb 0.28

GaMb 0.28 Goat polyclonal antibody against native Mb 0.28.
 MaG-HRP Mouse anti Goat immunoglobulin Horseradish Peroxidase conjugate
 Ref.: 205-035-108 Jackson Imm. Lab., Inc. (USA).

TD1 and GaMb 0.28 were concentrated by ammonium sulfate precipitation (40% w/v).

4. SOLUTIONS

1. PBS Phosphate Buffer Saline. pH 7.4. (10X)

For 1 l:	NaCl	80 g
	Na ₂ HPO ₄ , 12H ₂ O	29 g
	KH ₂ PO ₄	12 g
	KCl	2 g

2. Washing solution

0,1% Tween 20 in PBS

3. Carbonate buffer, pH 9.6

For 1 l:	Na ₂ CO ₃	1.59 g
	NaHCO ₃	2.93g

4. HRP-Substrate

Stock solution: 100 mg ABTS in 4,5 ml water.
 Citrate buffer 0.05M, pH 4

For 20 ml (Fresh mixture): 20 ml citrate buffer
 100 μ l ABTS stock solution
 20 μ l H₂O₂ 30%

5- Extracting solution

Glycine 0.3 M, pH 11.
 0.06% 2-Mercaptoethanol

5. PROCEDURE

1. Extraction of proteins

Pasta samples were ground in a KT30 grinder fitted at setting 2 (maximum particle size 250 μ m).

Ten ml of the extracting solution was added to 1 g of sample in a polypropylene tube, and stirred (rotary mixing) during 4 hr at room temperature. After centrifugation at 5000 g for 30 min the supernatant was diluted 40 times in PBS and kept overnight at 4°C.

2. Sandwich ELISA test

For all steps reactant volume was 0,2 ml per well. Between each step, wells were washed 5 times with washing solution.

The ELISA plates were coated with TD1 mAb at 3 g/ml in carbonate buffer overnight at 4°C. All next steps were performed at 37°C. After washing, a 5% w/v non-fat milk solution in carbonate buffer pH 9,6 was added to each well for 1 hr. Diluted pasta extracts in PBS (1/1000, 1/5000, 1/25000, ...) were then added to wells for 1 hr 30. Goat anti-Mb 0.28 was incubated at 20 g/ml for 1 hr 30 to reveal protein Mb 0.28. HRP-MaG conjugate (1/6000) was added for MaG. Peroxidase was detected by adding the HRP-Substrate. The reaction was stopped after 20 min incubation at 37°C by adding 10 μ l of a 10% w/v SDS solution . The absorbance of the wells was determined at 414 nm using the plates reader.

Annex IVa

Summary of the Research Done on Isoelectric Focusing of Albumin Specific Fractions

P. Resmini

**Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche
Università degli Studi di Milano, Via Celoria, 2, 20133 Milano, Italy**

1. OBJECTIVES

Determination of specific albumin fractions ("A", "B", "C") of common wheat and ("1") of durum wheat in pasta dried under elevated temperature conditions.

2. RESULTS OBTAINED

An improvement of the extraction procedure followed by IEF did not allow to detect the specific fractions of common wheat in pasta dried at high-temperature. It was possible to detect common wheat flour in pasta dried with milder drying cycles but the calibration curve depends on the drying conditions

RP-HPLC of salt-soluble specific proteins did not show significant results for pasta sample dried under high-temperature conditions.

3. CONCLUSIVE REMARKS

The results we obtained show that the more severe the drying conditions of pasta the stronger the difficulties for detecting common wheat flour. Taking these results into account, we think that any analytical method based on the evaluation of protein components will necessarily show low accuracy (markedly for the quantitation) due to the heating conditions which could be used. Because it is quite impossible to have as many standard samples as the used drying-conditions, in our opinion, it is imperative to study analytical parameters useful for evaluating the degree of the heat-treatment of pasta. These parameters could allow to correct the quantification of common wheat flour on the basis of the drying conditions.

Often drying cycles performed at the highest temperature involve lower costs of pasta manufacturing without increasing the final quality. On the contrary, pasta quality should be also high if the heat damage promoted by the drying conditions is kept low.

For the above-mentioned reasons, we think that it should be useful to clarify what pasta quality really means. Due to the different national legislations, we point out the necessity to develop methods allowing the detection of common wheat flour in high-

temperature dried pasta. This determination is probably more accurate if it can be related to the heat-damage evaluation.

Annex IVb

Development of Analytical Methods for the Determination of Common Wheat in Pasta Products Dried at Elevated Temperatures

P. Resmini

Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche
Università degli Studi di Milano, Via Celoria 2, 20133 Milano, Italy

and

N. Pogna

Istituto Sperimentale per la Cerealicoltura, Via Mulino 3, S. Angelo Lodigiano
(Milano), Italy

1. PRINCIPLE OF THE METHOD

Determination by means of isoelectric focusing (IEF) of albumin specific fractions ("A", "B", "C") of common wheat pasta dried under high temperature conditions.

2. SAMPLE PREPARATION

- Extraction with diluted acid solution of albumin specific fractions from pasta sample;
- Selective precipitation of albumin specific fractions in the range 0.8 - 1.6 M $(\text{NH}_4)_2\text{SO}_4$;
- Purification of the precipitated fractions by means of solid phase extraction (SPE).

3. IEF SEPARATION

- Electrophoretic separation of protein in the pH range 3.5 - 9.5;
- Trichloroacetic staining of the electrophoregram and quantification by densitometric scanning.

4. RESULTS

- The method allows to detect the specific fractions of common wheat albumins ("A", "B", "C") in Barilla pasta samples;
- The interpretation of the patterns for both durum and common wheat pasta is reported in **Fig. 1**;

- The densitometric scanning of the pattern is feasible for all the reported fractions as shown in **Fig. 2 to 9**;
- It is possible to detect till 1.5% of common wheat in Barilla A and B samples (**Fig. 3**); Barilla C samples can not be correctly analyzed by this method;
- The calibration curves (**Fig. 10**) are obtained by plotting the values of the area ratio: $\Sigma (A + B) / \Sigma (II + III + 1 + IV + V)$ against the known percentages of common wheat in Barilla B samples;
- The calibration curve seems to be dependent of the heating conditions applied during the pasta drying cycle. However, the electrophoretic pattern of low heat treated pasta (Barilla A samples) is quite different than the high treated ones.

5. CONCLUSION

The described method needs further improvements (extraction conditions, separation of the specific fraction and their evaluation) but it has been demonstrated that it is possible to detect common wheat in high heat-treated pasta on the basis of the same albumin specific fractions ("A", "B", "C") as in the case of the Italian official method.

Annex V

Durotest - Immunochemical detection of the common wheat specific albumin, *friabilin*

M. Bony

**Rhône-Poulenc Diagnostics Ltd, Montrose House
187 George Street, Glasgown G1 1YT, Scotland**

and

W. Stimson

**Head of Immunology Deptment, University of Strathclyde,
The Todd Centre, 31 Taylor Street, Glasgow G4 0NR, Scotland**

Assessment of Occurrence of Friabilin in Common Wheat

To establish if the amount of friabilin in common wheat varieties varied significantly, 31 samples of common wheat, incorporating 28 different varieties, were tested using Durotest P. **Table I** shows the absorbance values at 450 nm and the percentage coefficient of variance for each of the cultivars.

Table I

Cultivar	Absorbance at 450 nm	% Coefficient Variance
APOSTLE	1.687	4.8
APOLLO	1.713	4.8
AVALON	1.857	2.3
AVALON	1.673	2.2
AVALON	1.714	2.0
AVALON HEAT DAMAGED	1.559	4.2
AXONA	1.366	4.1
BOXER	1.768	2.3
BRIMSTONE	1.751	1.0
CAMP REMY	1.758	3.3
CANON	1.741	5.2
COURTOT	1.596	4.8
CWW/88/5	1.796	3.9
CWW-DEAN	1.686	3.3
DNS	1.712	2.7
FLORIDA	1.925	3.1
FRENCH FEED	1.542	4.7

FRENCH SUPERIOR	1.708	2.7
GALAHAD	1.493	3.3
HEREWARD	1.567	3.7
HORNET	1.688	3.5
HUNTSMAN	1.541	2.6
MERCIA	1.482	3.4
MISSION	1.595	1.7
PASTICHE	1.506	3.2
SLEJPNER	1.611	9.5
SUPERIOR NO.1	1.653	3.9
TORFRIDA	1.778	2.3
URBAN	1.479	3.4
WEMBLEY	1.318	4.2
NORMAN	1.605	4.1

(mean values of n = 8 determinations)

All of the 28 cultivars tested were found to contain a high level of friabilin as expected. None of the common wheat cultivars contained markedly less friabilin. The variation in absorbance values between different wheat cultivars was also very low, % CV = 8.2%.

Each wheat sample was extracted once and the extract applied to eight wells. The variability from well to well for each of the samples was very low with the highest % CV = 9.5%.

Assessment of Intravariability of Durotest P

To establish the intravariability of Durotest P, standard samples of different heat treatments were tested using the standard protocol. The results shown in **Table II** demonstrate that the percentage coefficients of variance are $\leq 10\%$ for all standards. Thus the intravariability for Durotest is $\leq 10\%$.

Table II

% Adulteration of Pasta	Heat Treatment	O.D. at 280nm	O.D. at 450nm	
			Mean N=6	% Coefficient of Variation
0	60°C	0.536	0.091	10.0
1-2		0.525	0.440	7.0
3-4		0.507	0.654	4.0
4-5		0.521	0.821	7.0
10-11		0.572	1.586	2.0
0	92°C	0.295	0.082	2.4
1-2		0.339	0.259	6.3

3-4		0.259	0.444	5.3
4-5		0.319	0.308	5.2
10-11		0.302	1.774	6.0
11-12		0.330	1.748	3.6
15		0.282	2.428	2.7
0	100°C	0.341	0.079	0.0
3		0.303	0.281	3.0
10		0.282	0.831	9.4

Effect of Heat Treatment of Pasta Samples on Results Obtained With Durotest P

Detection of adulteration of pasta with non-durum wheat often causes problems for manufacturers and retailers because the temperature at which pasta is dried can influence the accuracy of the results. Some pasta manufacturers treat pasta at very high temperatures to reduce the required drying time. In doing so, this denatures the proteins in the pasta which are normally used to determine the level of adulteration. In contrast, Durotest detects the protein friabilin, which is unaffected by the heat treatment of pasta.

It should be noted, however, that the extraction of friabilin from pasta becomes more difficult as the temperature of pasta heat treatment increases due to the formation of a more complex pasta. Therefore, we would recommend that when using Durotest, if the heat treatment of the pasta is known, pasta standards treated at the same temperature conditions are included in the assay.

If however, the temperature of pasta heat treatment is unknown, then the absorbance values at 280nm of the pasta extract can give an indication of the temperature of heat treatment. For example **Table II** shows the absorbance values at 280 nm obtained for the pasta standards heat treated at different temperatures. There is an obvious trend in the values obtained; for 60°C heat treatment the OD at 280 nm are ≈ 0.5 and for 92°C and 100°C heat treatment the OD at 280 nm ≈ 0.3 . Thus the absorbance value at 280 nm can be considered to be indicative of temperature of heat treatment.

Results With BCR Samples Using Durotest

The results shown below (**Table III**) were obtained from analysis of the BCR ring trial samples supplied by Dr Landi. The first batch of 10 unknown samples (labelled 1-10) arrived in April 1992 and a second batch consisting of 3 samples (labelled R, X and Y) was sent in September 1992.

Table III

Sample Number	Exact % of Common Wheat	Durotest Plate % Adulteration
---------------	-------------------------	-------------------------------

1.	3-4	2-3
2.	1-2	1-2
3.	8-10	7-8
4.	0-1	0
5.	4-5	4
6.	6-7	7
7.	8-10	6-7
8.	1-2	1
9.	0-1	0
10.	4-5	5-6
R.	0	0
X.	1.5	1-2
Y.	4.5	3-4

The Rhône-Poulenc Durotest plate format gave a very good estimation of the percentage of adulteration by common wheat. All the results differed by less than 1% from the reference values, except sample 7, which was slightly underestimated.

DURUM TEST

ELISA

Used for pasta and semolina samples; standards prepared by adulteration of durum wheat with quantities of common/hard wheat

- 1) 100 mg sample extracted in 0.5ml SDS/Tris buffer. Semolina standards 0%, 5%, 10%, 15%, 20%, 50%, 100% (from RHM).
- 2) Centrifuge samples and dilute 1/100 in Tris-buffered saline (TBS).
- 3) Add 100 μ l into ELISA plate incubate for 3 hr at 37 C.
- 4) Wash plate with TBS containing 0.5% Tween 20 (TTBS), dry plate.
- 5) Add antibody-enzyme conjugate, 1 hr at 37 C.
- 6) Wash plate with ms, dry plate.
- 7) Add enzyme substrate, 10 min in 20 C; stop reaction with acid.
- 8) Measure A₄₅₀

Dipstick

Used for semolina samples.

- 1) Spot 2,5 μ l samples from 100 mg semolina (standards used in ELISA, extracted in 0.5ml SDS/Tris) onto nitrocellulose strip.
- 2) Block remaining area of strip in casein solution.
- 3) Wash strip in TTBS x4.
- 4) Incubate in antibody-enzyme conjugate 20 min at RT.
- 5) Wash strip in TTBS x4.
- 6) Transfer to substrate staining solution for 1 min.
- 7) Rinse dipstick in water to stop reaction.

8) Record result following comparison with standards.

FRIABILINCharacteristics

Molecular mass:	14.7 kDa
pI:	9.5 (basic)
Amino acid composition:	average

Antibody

Monoclonal antibody:	F7F (IgG1)
Kd:	5×10^{-9} M
Specificity:	disulphide bridge region
Cell line:	high secretor, suitable for ascites production.

**Annex VI - Minutes of the meeting held in Brussels on
13 December 1990**

Next meeting : Friday, 21 June 1991 - PARMA (Italie)

1. Participants

M. Belliaro (BCR), Mc Carthy (Nottingham Polytechnics), Denoni (Università degli Studi di Milano), Feillet (INRA), Griffin (Nottingham Polytechnics), Landi (Barilla), Lumley (LGC, Teddington), Paraf (INRA), Resmini (Università degli Studi di Milano), Thibault (DG VI), Violle (Université de Montpellier).

2. Agenda

- Work completed during the period June-December 1990
- Future work and planning for the period January-July 1991
- Financial, legal and administrative matters.

3. Work completed (June-December, 1990)

Newsletters for period June-December 1990 were distributed by each group to all participants:

- immunochemical detection of common wheat gliadins in durum pasta (M. Griffin and K. Mc Carthy)
- electrophoretic determination of ω -gliadins (J.C. Autran)
- immunochemical detection and quantification of common wheat in durum pasta dried at high temperature (P. Violle and A. Paraf)
- determination of specific albumins ("A", "B", "C") of common wheat and ("1") of durum wheat in pasta dried under elevated temperature conditions (P. Resmini and N. Pogna).

The four reports were presented (summaries are given in **Annexes VI a,b,c,d**). The following points arised from the general discussion (see summary on **Table I**):

- a. The four programs are still promising and have to be pursued.
- b. The main obstacle to the practical use of the two methods based upon the determination of gliadin fractions is the origin - agronomic(?) and genetic - effect of common wheats. From this point of view, the two "albumin" methods have a better chance of success.

c. The protocol of some methods is (almost) available:

- ω -gliadin electrophoresis (J.C. Autran)
- ω - and γ -gliadin electrophoresis with and without HPLC (M. Griffin)
- albumin electrophoresis (P. Resmini)

Some works are still necessary to develop others:

- γ -gliadin immunochemical detection (M. Griffin)
- albumin immunochemical detection (P. Violle)
- albumin HPLC and immunoelectrophoresis (P. Resmini)

d. Respectively, J.C. Autran and M. Griffin consider that the quantitative solubilization (in their experimental conditions) of ω - (J.C.A) and specific γ - (M.G) gliadins is not affected by the drying temperature of pasta.

This is not yet demonstrated in the methods of P. Violle or P. Resmini. Nevertheless, very promising immunosera were developed by P. Violle to overcome this difficulty, while P. Resmini has good hope of elaborating a new and efficient method of "heat denatured" albumin extraction.

e. Exchange of materials between P. Violle and P. Resmini showed that:

Albumin C (P.R) = Albumin 0.28 (P.V)

Albumin A,B (P.R) (=) Albumin 0.19 (P.V)

4. Planning (January to July, 1991)

See **Table I**

a-Samples

ACTION

. Series A : Pasta samples have been prepared by (Barilla) and will be send to each groups (Milan, Montpellier, Nottingham) (each sample : 2 kg per participant) by January 4, 1991:

A. Landi
January 4

Drying technology a) 60°C

b) 92°C + 78°C

c) 100°C

Composition of samples

07+08) 100% of durum wheat

03+12) 98-99% of durum + 1-2% of common wheat

06+09) 96-97% of durum + 3-4% of common wheat

02+13) 95-96% of durum + 4-5% of common wheat

05+10) 89-90% of durum + 10-11% of common wheat

01+14) 88-89% of durum + 11-12% of common wheat
 04+11) 100% of common wheat

The list 01-07 is for pasta dried at 92° + 78°C
 and the list 08-14 for pasta dried at 60°C.

The drying flowsheets of these samples were distributed
 to all participants during the meeting.

Two other sets of samples will be prepared and sent
 to each groups:

ACTION

. Series B: pasta with 15, 25 and 50% of common
 wheat. Two drying technologies : 60°C, 92°/78°C.
 By February 1, 1991.

A. Landi
 February 1st

. Series C: blind pasta samples. Common wheat
 content and drying technology determined by
 Barilla in relation with BCR. By April 1st, 1991.

A. Landi
 J.J. Belliardo
 April 1st

b-Methods

A full description (as a standard) of the different methods
 will be distributed to participants by March 1st, 1991:

- ω-gliadin electrophoresis (J.C.A)
- γ-gliadin electrophoresis and HPLC (M.G)
- albumin immunoelectrophoresis (P.V)
- albumin electrophoresis (P.R)
- undetermined:
- gliadin immunoelectrophoresis (M.G)
- albumin HPLC and immunoelectrophoresis (P.R)

P. Resmini
 J.C. Aufran
 P. Violle
 M. Griffin
 March 1st

c-Analysis

Samples of series A and B will be analysed by each group
 and results sent to all participants by March 1st (P.R, J.C.A, M.G)
 or April 1st (P.V)

- ω-gliadin electrophoresis (J.C.A)
- γ-gliadin HPLC (M.G)
- albumin immunoelectrophoresis (P.V)
- albumin electrophoresis (P.R)

P. Resmini
 J.C. Aufran
 P. Violle
 M. Griffin
 April 1st

Samples of series C will be analysed by each group and results sent to all participants by June 1st, 1991

P. Resmini
A. Paraf
P. Violle
M. Griffin
June 1st

d-Next Meetings

The works by P. Violle (Montpellier) and by N. Pogna (Italy) have large similarities. A meeting will be organized by the two groups (A. Paraf, P. Violle, P. Resmini and N. Pogna) by February 15, 1991

P. Violle
A. Paraf
P. Resmini
N. Pogna
February 15th

J.J. Belliaro will decide the opportunity of holding a meeting in April to evaluate the results of the analysis of series A and B of pasta samples

J.J. Belliaro

Pending the agreement of Barilla, the next meeting of evaluation will take place in Parma, Friday 21 June 1991

5. Administrative aspects

The participants acknowledged the good management of the program by BCR office. No problem was raised.

To secure any further legal protection (patent) of the methods developed by any of the four groups an undertaking of secrecy was signed by all participants to whom copies of the undertaking were distributed.

The meeting was closed at 4:00 p.m.

P. Feillet

Annex VI a

Research group: Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche - Università degli studi di Milano (Italy) - (**Prof. P. Resmini**) and Istituto Sperimentale per la Cerealicoltura - S. Angelo Lodigiano (Italy) - (**Dr. N.E. Pogna**).

Summary of the work done from June till November 1990

During the last six months all the produced antisera were tested in order to check their specificity. These tests were performed using the Western Blotting technique on electrophoretic separations performed with different methods. Polyclonal antisera raised against protein "A" (anti-A), purified by EPE, showed a strong reaction with band 14-16 kDa in size (LMW albumins) in the SDS-PAGE patterns of total protein from common or durum wheats.

LMW albumins from pasta prepared from common wheat flour and dried at 90° C also interacted with anti-A, whereas LMW from high temperature (HT) dried durum wheat pasta showed a weak reaction. In order to obtain an antiserum with sufficient specificity for detecting adulteration of HT-dried durum wheat pasta, anti-A was purified by affinity chromatography using durum wheat albumins as ligands. In the same time we tried to better purify common wheat albumins "A", "B" and "C" by LPLC followed by preparative IEF

The results obtained suggest that the purified anti-A has sufficient specificity for the identification of common wheat LMW albumins and therefore was decided to produce monoclonal antibodies against protein "A".

Concerning the HPLC method, no important progresses were reached.

Annex VI b

Immunochemical detection of common wheat gliadin in durum pasta

K. McCarthy and M. Griffin

Work has progressed on the development of an anti-gliadin antisera which recognises γ -gliadin from all common wheat varieties and shows no cross-reactivity for durum wheat gliadin. The antibody preparation reacts very similarly on immunoblots to the preparation reported previously (γ -gliadin being the major protein recognised). This antisera was prepared using γ -gliadin isolated between 47-49 min on RP-HPLC of gliadin from an adulterated pasta. These adulterated gliadins probably contain a mixture of γ -gliadins from different common wheats which may explain the broad specificity of the antisera. Using this antisera it was shown on immunoblots that it is possible to detect gliadins found only in common wheat and absent in durum wheat. For quantitative purposes the affinity purified antisera was used to detect common wheat using a non-competitive ELISA where gliadin was bound to the microtiter well using poly-l-lysine. Initial results using this technique showed that common wheat gliadin can be detected, however, major amplification of the response is required if the method is to prove of use in analysis. Pure γ -gliadin was prepared using SP-Sephadex C-50 which is being used to raise larger quantities of anti- γ -gliadin antisera. The elution of ω -gliadin on RP-HPLC separation of total gliadin extracts was further improved using a Bio-Rad C3 column.

Annex VI c

Quantification of common wheat adulteration in pasta by immunodetection of soft wheat albumin proteins

P. Violle, A. Paraf and P. Feillet

We purified two α -amylase inhibitors (MB 0.19 and Mb 0.28) of common wheat and raised polyclonal antibodies to native and denatured (100° C, 10 min) proteins. After immunosorption against durum wheat proteins, we obtained specific common wheat antisera which reacted with native and denatured proteins.

Monoclonal antibodies were prepared against the Mb 0.28 protein. Three types of antibodies were obtained. Some reacted with common as well as with durum wheat proteins. Others reacted only with common wheat proteins. In this second group we found antibodies recognising either native protein alone or native and denatured Mb 0.28 protein.

We selected monoclonal antibodies of this last type to perform a sandwich ELISA test to detect adulteration in pasta. Preliminary experiments showed a high sensibility of the test. However, the procedure of protein extraction and ELISA test must be improved to obtain a good specificity and repeatability.

Annex VI d

Electrophoretic determination of ω -gliadins

J.C. Autran, INRA, Montpellier

Summary of the Newsletter for period June-December 1990

Experimental conditions for analysis of unknown pasta samples were drafted. These conditions include: cooking, freeze-drying, gliadin extraction, PAGE electrophoresis, determination of common wheat content based on the densitometric scanning of the genome D-specific ω -gliadin bands, referring to a set of standards.

It was confirmed that there is no significant difference whether the standards are prepared from semolina-flour mixtures, from spaghetti, or from sheeted doughs, and that undergo low or high temperature drying.

The linearity of the relationship between densitometric scanning and common wheat ratio in the concentration range used was demonstrated. The coefficients of variation for various ranges of common wheat content were determined. It was confirmed that preliminary heat treatment by cooking can erase differences in the thermal history of the samples from both pasta or other type of product (couscous, semolina, reground pasta).

The method was tested with various industrial products that were likely to be adulterated by bread wheat. The major limitation observed was the variability of composition and content in ω -gliadins among wheat cultivars. The major advantage of the method is the remarkable heat resistance of ω -gliadins, that allows reliable determinations even in extremely heat-denatured products.

Annex XI - Literature

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