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## **AAC COMMENTS TO THE FDA on “GLUTEN-FREE” Public Meeting – August 19**

As suppliers of “gluten free” food ingredients and encouraged by the ongoing revision of the Codex Standard for Gluten-Free Foods and by the provisions on allergen labeling in the E.U., the European Cereal Starch Industry Association (AAC) has made a great effort during the last years to improve the knowledge regarding gluten content and nitrogen compounds of wheat starch and wheat starch derivatives.

Therefore, the AAC would like to submit information and comments to FDA regarding Gluten content and analyses procedure for wheat starch and wheat starch derivatives in Europe.

### Gluten content and Nitrogen compounds of Wheat starch:

In 1981 Codex Alimentarius defined “Gluten-Free” Foods as products containing less than 0.05 g nitrogen per 100 g on dry matter. However many products, including wheat starch, contain considerable amounts of non-gluten endogenous proteins and of non-protein nitrogen compounds which are also measured by total nitrogen determination.

The Codex Standard currently under revision aims to define a specific gluten threshold for gluten free foods based on gluten tolerance in celiac patients. The revision of the Codex Standard for Gluten-Free Foods was put on hold in 2001 until a reliable method for the determination of gluten and scientific data on the tolerable intake of gluten by celiac people become available. In the current proposal, two levels of gluten are considered for gluten-free foods: maximum 200 ppm/ds for products containing derivatives of gluten-containing cereals, and maximum 20 ppm/ds for naturally gluten-free foods.

Does wheat starch comply with those levels?

During 4 years, AAC collected data from the major European Producers on N content of wheat starch. The N content is one of the quality parameter in the production of wheat starch. The average content is 0.036 % N and all the values are below 0.056 % N. From those data, it can be concluded that 80-85% of the industrial wheat starch complies with the current Codex specification (0.05 g N /100 g D.S). The remaining 15-20% is only slightly higher.

Facing the revision of Codex Standard and the provisions on allergen labeling in the E.U., AAC members have conducted extensive analytical studies in order to clarify what is the contribution of different compounds containing nitrogen in total nitrogen content of wheat starch and wheat starch hydrolysates (see study in annex I as part of the file submitted by the AAC in the context of the provisions for exemption of certain products from allergen labeling in the E.U.).

For wheat starch, it has been shown that 60% of total N originates from lipid compounds and only 30% from proteins. The major part of granule starch proteins is an enzyme involved in biosynthesis of starch, namely Granule Bound Starch Synthase.

Gluten in wheat starch was measured using the R5 Enzyme-Linked ImmunoSorbent Assay (ELISA) method. The gluten contents were principally between 50 and 150 ppm with a maximum value of 242 ppm.

Therefore, taking into account these results, the European Cereal Starch Industry Association is confident that a substantial part of wheat starch produced in Europe complies with the limit of 200 ppm gluten as proposed in the revised draft Codex Standard for Gluten-free foods.

#### Wheat starch derived products: glucose syrups and maltodextrins

Wheat starch derivatives are obtained through partial or total hydrolysis of wheat starch. During the manufacturing process wheat starch derivatives undergo efficient purification steps including protein removal by active carbon treatment and ion exchange.

**The nitrogen content of wheat starch derivatives is ranging from about 15 ppm (detection limit of the method) to a maximum of 150 ppm which gives maximum 1000 ppm expressed as apparent protein content (Nx6,25).**

A study conducted using amino acid analyses and mass spectrometry demonstrated that only trace amounts of protein (1-40 mg/kg) are detectable in wheat starch glucose syrups and maltodextrins. Part of these proteins is peptides that arise from degradation of gluten. The difference between apparent protein and true protein in wheat starch derivatives is explained by N-containing lipid residues.

By applying the sandwich and competitive R5 ELISA method, the response was below the detection limit of 3.1 and 2.4 ppm respectively.

#### Method of determination of gluten:

Most of the available methods for quantitative determination of gluten in foods are immunological.

Two types of commercial ELISA tests kits were used for several years: 1) a monoclonal antibody raised against an omega-gliadin peptide from the Australian wheat variety Timgalen, originally developed by Skerrit. The method has been approved as standard by AOAC (AOAC Official Method 991.19). 2) a polyclonal antibody raised against a mixture of alpha, beta, gamma and omega gliadin fraction purified from a mixture of fifteen different German wheat varieties originally developed by Riedel de Haen.

As it could be expected the reactivity of these two antibodies is different. Furthermore, the protein preparations included in each kit as standards being also slightly different in composition as indicated above, any direct quantitative comparison between the two methods is impossible.

A more recent second-generation approach, followed by several academic laboratories consisted in raising monoclonal antibodies directed against chemically synthesized peptides, the sequence of which are deduced from amino acids sequence analysis of cloned gliadin genes. The principle

is to choose a short non-repetitive sequence, which will allow the recognition of a single type of molecule. Although quite appealing in theory, this approach has led to low sensitivity tests which could not meet the requirements of an industrially applicable method.

Méndez et al. (Unidad de Gluten, Centro Nacional de Biotecnología, Madrid, Spain) have studied a monoclonal antibody, identified as R5. This method has been developed within the Prolamin Working Group. It uses a new monoclonal antibody, identified as R5 and originally isolated by Mendez. R5 recognizes several and small repetitive epitopes as QQPFP, LQPFP,QLPYP,QLPFP, QQTFP,PQPFP, QQPYP and PQPFP in gliadins.

R5 has been recently used as the base for two new kits developed by Ingenasa, a Spanish company, and R-Biopharm. The R5 antibody has a sensitivity of 1.5 ppm for gliadins (3 ppm for gluten) and it allows equivalent recognition of wheat, barley and rye glutens. Valdés et al. also showed by Western blotting the reactivity of R5 towards enzymatically or heat-modified wheat prolamines. Immer et al. (2002) performed a ring test with 20 laboratories on the R5 ELISA and they concluded that the method is able to detect gliadin in the range of 2-5 ppm using the Prolamin Working Group gliadin standard.

The classical sandwich ELISA R5 has some limitations that still have to be solved, and this method is hardly applicable to wheat starch hydrolysates and therefore should be considered with particular caution when applied to these ingredients or to food products containing them. The competitive R5 ELISA method, which is under development, aims to solve these problems. This should be a significant improvement, enabling us to get a practical and reliable method for determination of gluten in such products, but the method still needs to be validated against a standard reference method. Several commercial kits are now available based on the R5 antibody such as the Ridascreen gluten kit (R-Biopharm, Germany) and the Ingezim gluten kit (Ingenasa, Spain).

The Mendez's method has been proposed and submitted to Codex Alimentarius for approval. In April 2005, the Codex Committee on Methods of Analysis and Sampling has agreed to endorse temporarily the R5 ELISA method for the determination of gluten as a Type I method.

### The European starch industry's position on Gluten-free

The revision of the Codex Standard for Gluten-Free Foods was postponed in 2001 until a reliable method for the determination of gluten, and scientific data relating to the tolerable levels of gluten for coeliac patients became available. Based on the current proposal, two levels of gluten are being considered for gluten-free foods: maximum 200 ppm/ds for products containing derivatives of gluten-containing cereals, and maximum 20 ppm/ds for naturally gluten-free foods. Both categories would be labelled as "gluten-free".

The maximum level of 200 ppm gluten is already being commonly used on the market for wheat-based gluten-free foods, and the same limit is also applicable to the wheat starch ingredient used in these foods. Recent data on the gluten content of wheat-based gluten-free foods show that these products generally contain about 100 ppm gluten or less. This is in line with the 200 ppm limit value used by products on the market for the following reasons:

- In order to be able to guarantee maximum 200 ppm gluten for wheat starch, a sampling and analytical error needs to be taken into account by the starch producer.
- Wheat-based gluten-free foods contain a maximum of 70-80% wheat starch.

- As indicated above, the gluten content of industrial wheat starch is generally between 50 and 150 ppm.

The upper limit for gluten-free foods will need to take into account data on tolerable levels for daily intake of gluten by coeliac patients, and data on exposure to gluten through the use of wheat-based gluten-free foods. Based on the data available today, a gluten content of wheat based gluten-free foods of about 100 ppm or less will result in daily exposure levels to gluten that are acceptable for a large majority of coeliac patients. A second category of gluten-free foods, guaranteeing lower gluten content, will be suitable for the most sensitive coeliac patients.

The cereal starch industry therefore believes that the maximum level of gluten in wheat starch can be maintained at 200 ppm, as proposed in the revised draft of the Codex Standard for Gluten-Free Foods. This will reinforce the present situation of the market, which offers wheat-based gluten-free foods containing about 100 ppm gluten or less.

One category of gluten-free foods with a low level of gluten acceptable for all coeliac patients would exclude the use of wheat starch in gluten-free foods.

Finally, it is important that a reliable and generally accepted reference method for the determination of gluten becomes available as soon as possible. Until then, the starch industry has no other choice but to continue using N as a parameter within the context of gluten-free product quality, even if it is not directly related to the protein and gluten content in starch and starch derivatives.

#### References:

AAC (2004) Gluten-free: the starch industry's view. Proceedings of the 19<sup>th</sup> meeting of the Working Group on Prolamin Analysis and Toxicity, Prague, 30 september-3 October 2004. Editor: Martin Stern, p. 165-169.

AOAC Official Method 991.19 Gliadin as a measure of Gluten in Foods – Colorimetric Monoclonal Antibody Enzyme Immunoassay Method First Action 1991, Final Action 2001

Ferre S., Garcia E, Méndez E. Measurement of hydrolysed gliadins by a competitive ELISA based on monoclonal antibody R5: analysis of syrups and beers. Proceedings of the 18th meeting of the Working Group on Prolamin Analysis and Toxicity, 2-5 October, Stockholm, 2003

Immer U, Vela C, Méndez E, Jensen F. PWG collaborative trial of gluten in gluten-free food through "Cocktail ELISA". Proceedings of the 17th meeting of the Working Group on Prolamin Analysis and Toxicity, 3-6 October 2002, Verlag Wissenschaftliche Scripten, Zwickau, Germany 2003.

Valdés I, García E., Llorente M., Méndez E. Innovative approach to low-level gluten determination in foods using a novel sandwich enzyme-linked immunosorbent assay protocol. *European Journal of Gastroenterology & Hepatology* 2003;15:465-474

**IDENTIFICATION AND QUANTIFICATION  
OF RESIDUAL NITROGEN COMPOUNDS  
IN WHEAT STARCH AND WHEAT STARCH HYDROLYSATES**

**Roquette Frères , April 2004**



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## **I Introduction**

A number of minor non-carbohydrate constituents (lipids, protein and minerals) are present in wheat starch. The efficiency of separation of wheat starch and gluten in starch industry is very high. However, it is well known that traces of nitrogen containing material remain in the most pure starch. From an AAC survey on industrial wheat starches, the total nitrogen content ranges from 0.026 % to 0.058 %, the average value is 0.038 %.

The nitrogen (N) is generally considered to be present as protein, but it may be even more important part of lipids. In 1992, *Skerritt et al* showed that the first 0.25 % (in absolute value) of Nx5.7 in wheat starch is associated to non-gluten protein and to nitrogen contained in phospholipids.

These phospholipids, containing nitrogen, are the major lipids in wheat starch. Non-gluten proteins are known as starch granule associated proteins (SGAP). They are divided into two groups: the surface SGAPs and the internal SGAPs.

For starch containing from 350 to 400 ppm (= mg/kg) of nitrogen, it was reported that 30-100 ppm of N arise from surface SGAPs, 130-150 ppm of N from internal SGAPs and 140-200 ppm of N from lipids (*Sulaiman and Morrison, 1990*). Gluten represents only a very small part of protein in wheat starch (from 1 to 10%) (*Skerritt et al, 1990*).

During the production of wheat starch hydrolysates (glucose syrups, dextrose and maltodextrins), efficient purification processes are used to remove the major part of impurities of wheat starch. Final nitrogen content commonly reaches a value far lower than 150 ppm. But no literature is available about the nature of these residual nitrogen compounds present in wheat starch hydrolysates (WSHs).

Because of the lack of specificity of total nitrogen determination using the Kjeldahl method, it is impossible to distinguish routinely proteins nitrogen and lipids nitrogen. Specific techniques should be applied. This report aims to review some results obtained in our lab that allowed to identify and to quantify major nitrogen containing impurities in representative industrial wheat starch and WSHs. Nitrogen balance emphasizes that lipids and lipid hydrolysis compounds represent the most important part of nitrogen containing impurities in those products.

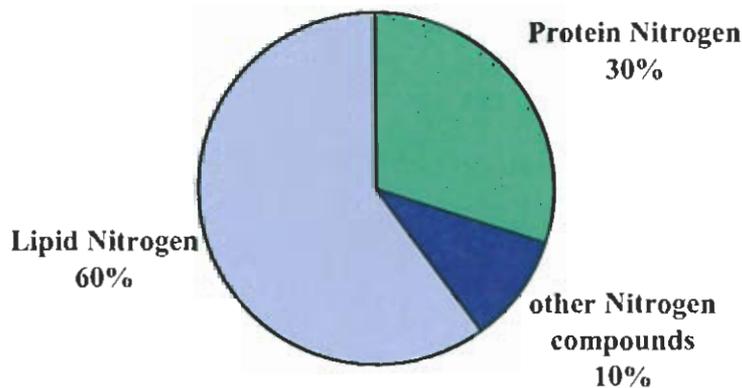
## II Nitrogen containing compounds in wheat starch :

### II -1 Nitrogen balance in wheat starch :

A four years survey conducted by AAC on nitrogen content of wheat starch shows that the average nitrogen content of starch is 0.038% (0.026 – 0.058%). Please see chapter 3.2.1. in the AAC notification.

Nitrogen balance of wheat starch can be presented as follows (see results further on):

*Figure n°1: Nitrogen balance of wheat starch*



### II- 2 Residual wheat starch lipids

Total lipids content in wheat starch ranges from 0.6 to 1% (internal data).

*Acker et al* were the first to establish that the principal lipids in wheat starch granules are lysophospholipids (*Acker et al 1967*). According to *Morrison*, lipids consist of over 90 – 94% lysophospholipids, 6-10% free fatty acids and traces of monoacyl lipids (*Morrison, 1988*).

The most important lysophospholipids are:

- LPC (Lysophosphatidylcholine)
- LPE (Lysophosphatidylethanolamine)
- LPG (Lysophosphatidylglycerol).

The two first (LPC and LPE) have one mole of nitrogen per mole of lipid (see formula in Annex 3).

LPC is the major lysophospholipid (~ 86%). LPE represents 10% of lysophospholipids and LPG 4%.

Considering a 520 g/mole average lipid molar mass, 0.6 to 1% of lipid content in wheat starch is equivalent to 160 ppm up to 270 ppm nitrogen in wheat starch.

Analyses and results:

Lipids are extracted with solvent (propanol/water) and quantified by weighing.

Lipids are then identified by HPLC. The chromatographic separation was carried out with a silica column bound to an evaporative light scattering detector. The compounds are eluted with a chloroform /methanol/ ammonia solution.

Total nitrogen analysis is carried out following the Kjeldahl procedure.

Amino acid determination is performed by HPLC (see Annex 1 for description of the methods).

Amount of lipid extracted, nitrogen content of wheat starch and nitrogen content of lipid extract are indicated in the table n°1 below.

*Table n°1: Nitrogen content of Wheat starch and wheat starch lipid extract*

	Dry matter %	% extract (propanol/water extract)	HPLC Identification	NMR Lipids estimation	Total nitrogen content (Kjeldahl) ppm / cp	Protein nitrogen ppm / cp (from amino acids analysis)	Amino acids ppm / cp
Wheat Starch	87.1			lipids (~1%)	344	83	743
Starch after lipid extraction	89.0			lipids (~0.1%)	146	82	733
propanol/water extract = lipid extract	-	0.9 %/DS (containing 2.1% of N)	Lyso-phospholipid		189	-	-

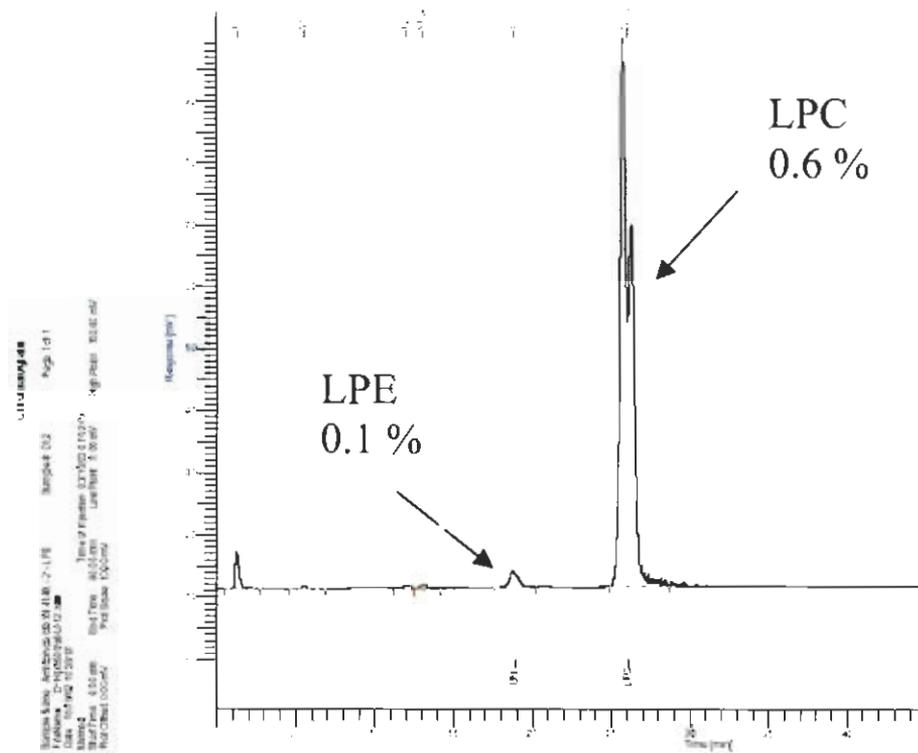
From wheat starch containing 344 ppm nitrogen (N), 0.9 % of lipids are extracted with propanol/water solution. This lipid extract contains 2.1% of nitrogen which represent 189ppm N considering the initial starch.

Nuclear magnetic resonance spectrometry (NMR) shows that 0.1 % of lipids is not extracted, which is about 25 ppm N/ starch.

As a conclusion from this study, we can consider that 62% of total starch nitrogen is due to the lipid compounds ( $= ((189+25) \times 100 / 344)$ )

The nature of nitrogen containing lipids, LPC and LPE, has been confirmed by HPLC carried out on lipid extract (see *figure n°2*).

**Figure n°2:** Principal lipids in wheat starch, LPC and LPE  
(LPC content in wheat starch is 0.6% LPC and 0.1% LPE)



## II- 3 Residual wheat starch proteins

The residual proteins of the wheat starch granule range from 5 to 149 Kda and can be classified into surface SGAPs and internal SGAPs according to their extractability from starch granules (*Skerritt et al, 1990*).

Electrophoresis of internal SGAPs, solubilized from starch swollen in a heated extraction buffer containing SDS, shows five discrete bands ranging from 59 to 149 Kda. Among the internal SGAPs of wheat, the major protein, 59 or 61 Kda, is the product of waxy gene and is called SGBSS I ( waxy protein or GBSS)(*Baldwin, 2001*).

The lowest molar mass proteins are surface proteins and range from 5 to 30 Kda. One type of those proteins has received specific interest, the 15 Kda polypeptides called "friabilin" (with the puroindolines as a major sub-group). It has been shown that puroindolines are the only protein species that interact with lipids (*Baldwin, 2001*).

In typical starch samples, total nitrogen content (350-400 ppm N) consists of 30-100 ppm surface proteins and 130-150 ppm N internal proteins. It has been proposed by *Rahman et al (1995)* that all the major associated proteins are likely to be involved in starch biosynthesis.

The classical analyses (electrophoresis, amino acid analysis, nitrogen determination, ELISA test) carried out aim to point out that the larger part of proteins in wheat starch are non-gluten proteins (see Annex 1 for description of the methods).

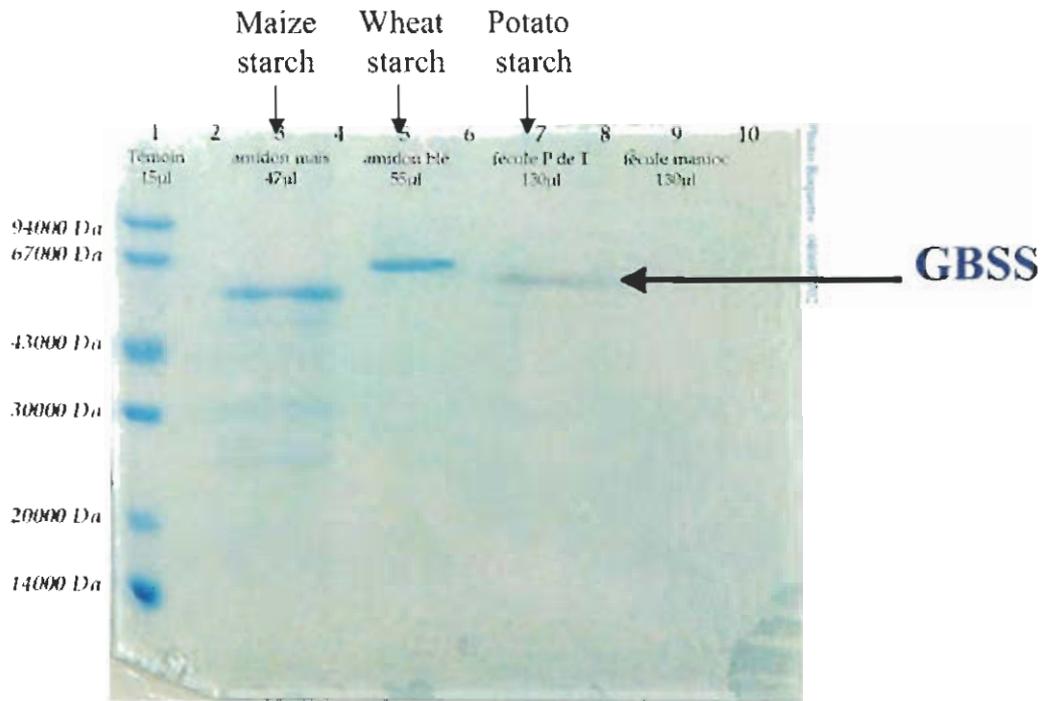
### II-3-1 Electrophoresis:

The extraction of proteins from wheat starch is performed following the procedure of *Vos Scheperkeuter (1986)*: the granules are gelatinized in a SDS/ beta-mercaptoethanol solution. After centrifugation, the supernatant is concentrated and put on electrophoresis gel (SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis). The procedure was applied on maize, wheat and potato starch.

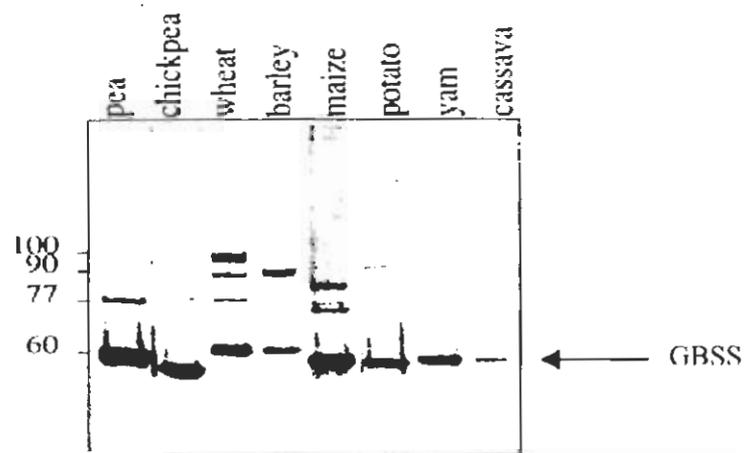
All species give a major band at about 60Kda that corresponds to the GBSS (granule bound starch synthase or SGBSS I) (see *figure n°3*). The inolar mass of the wheat starch GBSS is higher than the others.

This result is in accordance with other electrophoresis patterns presented in literature (*Denyer et al, 1996*) (see *figure n°4*). Higher molar mass protein (from 77 to 149 Kda) were not visible on our electrophoresis because of weak amount extracted of those proteins.

**Figure n°3: Electrophoresis of maize starch, wheat starch and Potato starch**



**Figure n°4: Electrophoresis (from literature)**



**Figure 1 Starch granule-bound proteins from cereals/beans (pea, chickpea), endosperms**

### II-3-2 Amino acid analysis

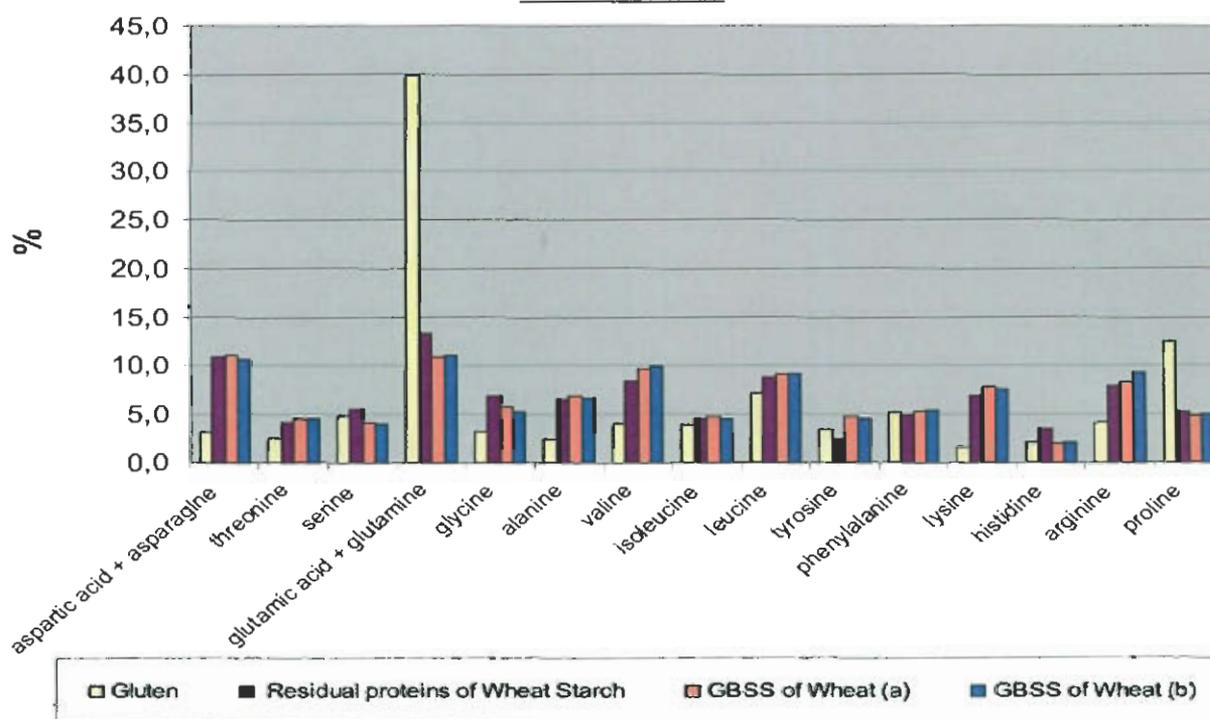
The amount of true protein of wheat starch, calculated from four total amino acid analyses, is 667 ppm. It represents roughly **100 ppm of nitrogen** (see table in Annex 2).

The average value of total nitrogen content of these four wheat starches is **322 ppm**, therefore about only 30 % of total nitrogen originates from proteins. 70% of nitrogen comes from other compounds mainly lipids (LPC and LPE).

Usually, food industry calculates the protein content from the total nitrogen determination using the 6.25 factor: therefore, in the case of wheat starch, the  $N \times 6.25$  value ( $322 \times 6.25 = 2010$  ppm) corresponds to an “apparent protein” value that strongly overestimates the *true* protein content (667 ppm).

The relative amino acid composition of starch proteins can be compared to the composition of GBSS (=SGBSS I) from literature and to gluten (see *figure n° 5*). The composition of protein from starch is very close to the composition of GBSS. This confirms the electrophoresis observation. GBSS is the principal protein of wheat starch. Gluten, characterized by a high glutamine<sup>1</sup> content, stands for only a very small portion of protein (<10%).

**Figure n°5: Amino acid composition of residual proteins from wheat starch compared to Gluten and GBSS**



<sup>1</sup> Glutamine is converted into glutamic acid after hydrolysis for total amino acid analysis

## **II – 4 Conclusions:**

Nitrogen compounds of wheat starch are:

- In the first place : **lipids** mainly LPC, 60% of total nitrogen is explained by the presence of lipids
- In the second place: **proteins**, mainly GBSS that represents 30% of total nitrogen.

Gluten represents only a faint part of starch nitrogen containing residues as shown in the Méndez report (please see chapter 3.2.3 in the AAC notification), less than 10% of residual proteins in well-refined wheat starch.

### **III Nitrogen containing compounds in wheat starch hydrolysates (WSHs)**

Very few literature is available on impurities in WSHs (glucose syrups, maltodextrins and dextrose). The residual nitrogen content is evaluated by Kjeldahl or Dumas methods in starch industry . The resulting N numbers are converted into “apparent proteins” using the 6.25 factor.

The range of nitrogen content is below 100 ppm, therefore roughly below 600 ppm in term of “apparent protein” (see results below).

Today starch producers are requested to provide accurate estimates of proteins, especially gluten. But up to now, no reliable method is available. Also Elisa tests have shown their limitations for hydrolysed products.

In addition to the study using advanced mass spectrometric technique for characterizing proteins and peptides (see P. Ferranti’s report), a further study has been carried out to get better knowledge on non-protein-compounds containing nitrogen in WSHs and to evaluate the classical amino-acid HPLC procedure as a possible technique to determine the *true* protein content in hydrolysates.

This study has shown using both NMR and amino acids HPLC that the major part of nitrogen does not come from proteins but mostly from lipid hydrolysis compounds.

**The true protein content is less than 100 ppm in all samples of WSH (<15 ppm of protein nitrogen).**

#### **III-1 Nitrogen balance**

The following table summarizes the results obtained from the study presented hereafter:

*Table n°2: Nitrogen balance in WSHs*

	<b>WSHs (maltodextrins and glucose syrups)</b>	<b>Technique</b>
<b>Total nitrogen</b>	<b>&lt; 100 ppm</b>	<b>Kjeldahl</b>
<b>Nitrogen from true protein</b>	<b>&lt;15 ppm</b>	<b>Amino acid HPLC</b>
<b>Nitrogen from lipid residues</b>	<b>&lt; 90 ppm</b>	<b>NMR, Phosphorus SAA</b>

### Samples:

The analyses were carried out on a number of samples, all provided by AAC members and representative from the major European wheat starch producers (*Amylum, Cerestar-Cargill, Chamtor, Roquette, Syral*):

***Table n°3: List of samples***

<b>Product</b>	<b>Process</b>	<b>Raw material</b>
<u>Wheat glucose syrup:</u>		
1) GLU/A/4	acid	wheat
2) GLU/A/7	enzymatic	wheat
3) GLU/B/5	acid	wheat
4) GLU/B/8	enzymatic	wheat
5) GLU/C/2	enzymatic	wheat
6) GLU/C/6	enzymatic	wheat
7) GLU/D/3	enzymatic	wheat
8) GLU/D/11	enzymatic	wheat
9) GLU/C/13	enzymatic	wheat
10) GLU/D/2	enzymatic	wheat
11) GLU/E/1	acid	wheat
12) GLU/E/14	enzymatic	wheat
13) GLU/F/9	enzymatic	wheat
14) GLU/F/10	enzymatic	wheat
<u>Maltodextrin:</u>		
15) MAL/A1	enzymatic	wheat
16) MAL/A4	enzymatic	wheat
17) MAL/B2	enzymatic	wheat
18) MAL/B3	enzymatic	wheat
<u>Dextrose:</u>		
19) DEX/A1	enzymatic	wheat
20) DEX/B2	enzymatic	wheat
21) DEX/B3	enzymatic	wheat

A complementary set of 29 wheat maltodextrins has also been investigated through NMR study.

### **III- 2 True protein content in WSHs**

Amino acid HPLC is a very sensitive method for protein quantification: usually, 1 ppm of each free amino acid can easily be detected and quantified. However, the hydrolysis of traces of proteins in presence of carbohydrate is rather difficult. Extraction of protein residues from carbohydrate matrices especially starch hydrolysates is time consuming. It is impossible to perform this procedure routinely (see P. Ferranti's report).

During the current study, the feasibility of performing direct hydrolysis of glucose syrups and maltodextrins has been investigated.

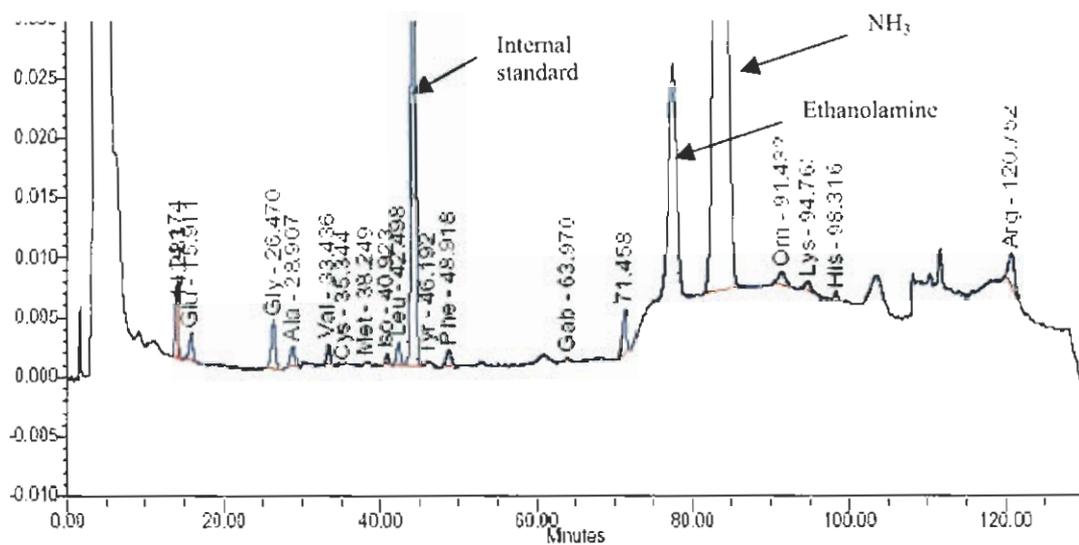
Therefore, as a consequence of the presence of carbohydrate degradation products after the protein hydrolysis step, interference “noise” occurs on the chromatogram baseline increasing the detection limit.

Despite this problem, a validation process has been run. The detection limit was estimated at **100 ppm** (sum of each amino acid) and the quantification limit at 150 ppm. (See example of chromatogram obtained with a maltodextrin, *figure n°6*)

Amino acid HPLC determination was conducted to compare results of true protein content with Nx6.25 content on WSH from AAC (see *table n°3*). **All the samples contain less than 100 ppm of true proteins.**

Similar results have been obtained with 50 other samples of wheat based maltodextrins and glucose syrups (internal data).

*Figure n°6: example of chromatogram of amino acid analysis obtained with a maltodextrin*



*Table n°4: Nitrogen, amino acids, phosphorus and nitrogen from ethanolamine in WSHs*

AAC Reference	Nitrogen (Kjeldahl)		Amino acid ppm / cp	Phosphorus ppm / cp	N from Ethanolamine ppm / cp
	Nitrogen ppm / cp	Nx6.25 ppm / cp			
GLU/A/4	33	206	<100	57	-
GLU/A/7	32	200	<100	56	-
GLU/B/5	ND*	-	<100	<1	ND**
GLU/B/8	ND*	-	<100	5	0.2
GLU/C/2	41	256	<100	86	3.8
GLU/C/6	17	106	<100	22	1.9
GLU/C/13	19	119	<100	11	0.8
GLU/D/3	46	288	<100	51	5.3
GLU/D/11	78	488	<100	134	6.2
GLU/D/12	10	63	<100	6	0.6
GLU/E/1	21	131	<100	39	1.6
GLU/E/14	34	213	<100	57	3.4
GLU/F/9	<15	<100	<100	<1	ND**
GLU/F/10	16	100	<100	20	1.6
MAL/A/1	<15	<100	<100	5	ND**
MAL/A/4	34	213	<100	51	5.7
MAL/B/2	91	569	<100	168	6.9
MAL/B/3	84	525	<100	155	4.8
DEX/A1	ND	-	<100	3	ND**
DEX/B/2	<15	<100	<100	2	ND**
DEX/B/3	<15	<100	<100	<1	ND**

ND\*: non-detected; Limit of detection 5 ppm  
 ND \*\*: non detected ; limit of detection 0.1 ppm

### III- 3 Non-protein nitrogen containing compounds in WSHs

#### III-3-1 Quaternary ammoniums by $^1\text{H}$ -NMR spectrometry

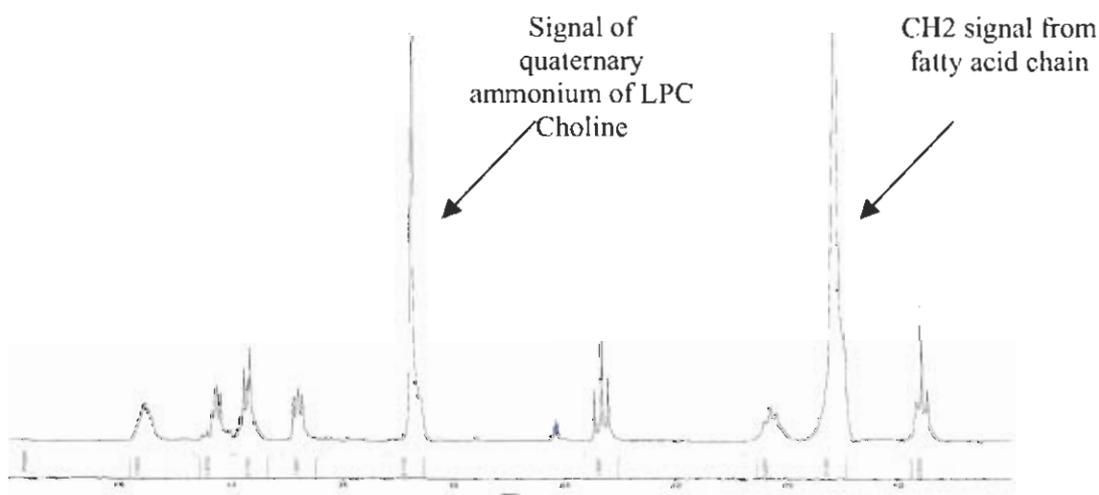
The difference between total amino acid content and  $\text{Nx}6.25$  strongly suggests that in addition of traces of proteins, other nitrogen containing compounds are present in the samples (see *table n°4*).

From study of the wheat starch, it is concluded that lipid is also be present in WSHs because the main source of nitrogen in wheat starch comes from LPC.

Lysophospholipid extraction from WSH with butanol, following the procedure of *Konieczny-Janda et al* (1991), shows that only a small part of entire LPC is present in maltodextrin samples. In an internal analysis with the procedure, less than 5 ppm of nitrogen is explained by LPC presence in a maltodextrin sample containing 77 ppm of nitrogen.

Starch Lipids are monoacyl lipids that means that one fatty acid is bound per molecule. With NMR, pure LPC gives two principal signals due to quaternary ammonium (choline residue) and fatty acid (see spectrum of pure LPC, *figure n°7*; see NMR procedure, Annex 3).

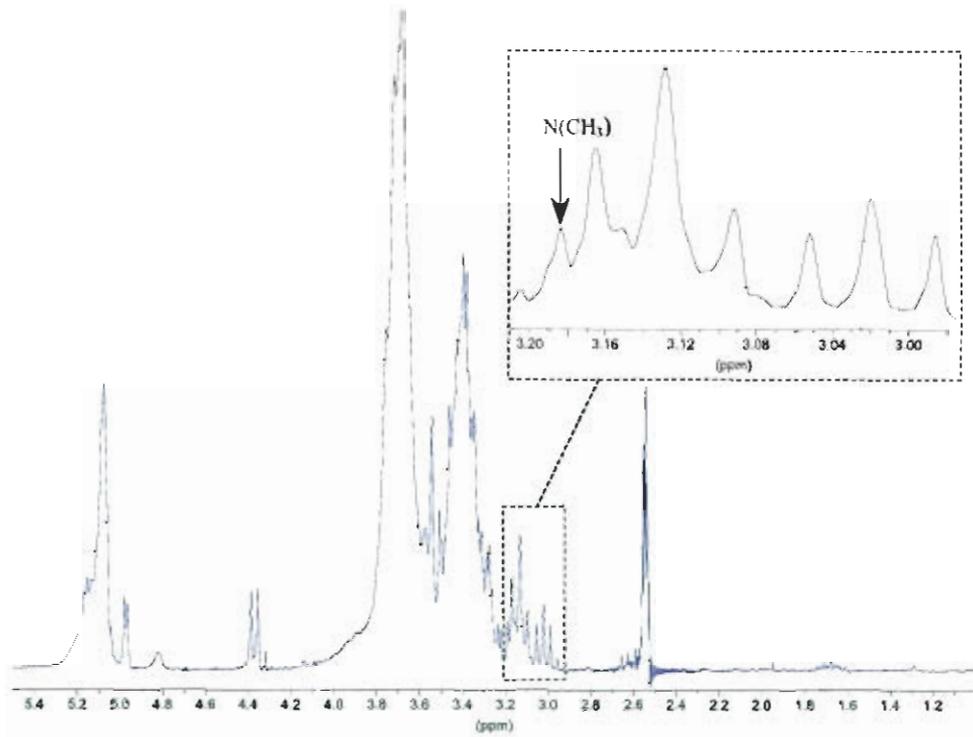
***Figure n°7: NMR spectrum of pure LPC***



Specific signals from lipids were searched with NMR directly in maltodextrin. A weak signal from quaternary ammonium at 1,3 ppm was pointed out (see spectrum, *figure n°8*) However, no signal from fatty acid was detected.

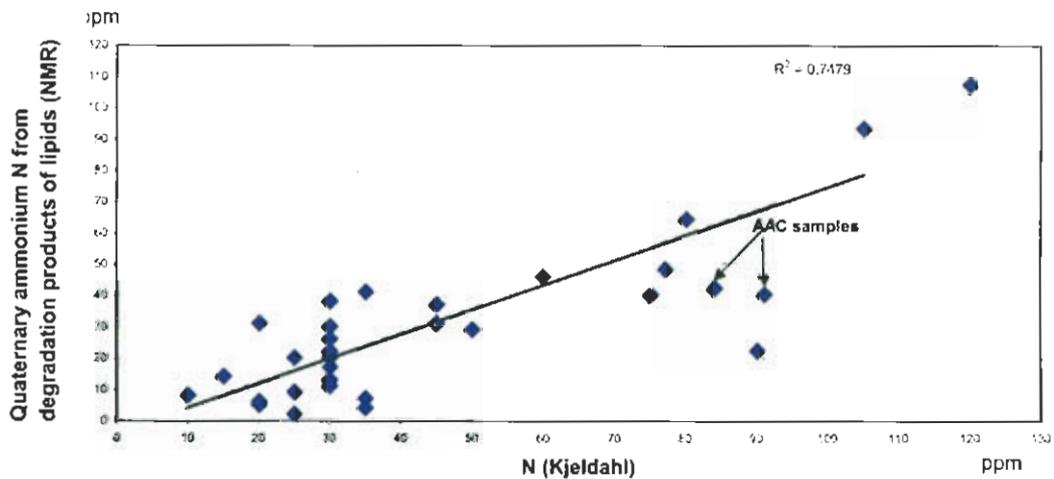
**This indicates that the majority of lipids in WSHs are compounds coming from lipid hydrolysis in maltodextrin.**

*Figure n° 8: NMR spectrum of maltodextrin*



Using a “spiking” procedure with LPC, a quantitative approach has been developed, allowing to estimate nitrogen from lipid hydrolysis compounds in maltodextrin. The relationship between quaternary ammonium nitrogen (NMR) and total nitrogen (Kjeldahl) is shown in the *figure n°9*.

*Figure n° 9: Total N vs Quaternary ammonium N from degradation products of lipids (ppm)*



It can be concluded from this study that **30 to 90 % of nitrogen present in wheat based maltodextrins is coming from lipid hydrolysis compounds, depending of the total nitrogen content (figure n°9).**

Unfortunately, because of strong low molecular mass carbohydrate interferences, it was not possible to detect the quaternary ammonium signal in WSH.

### III-3-2 Phosphorus

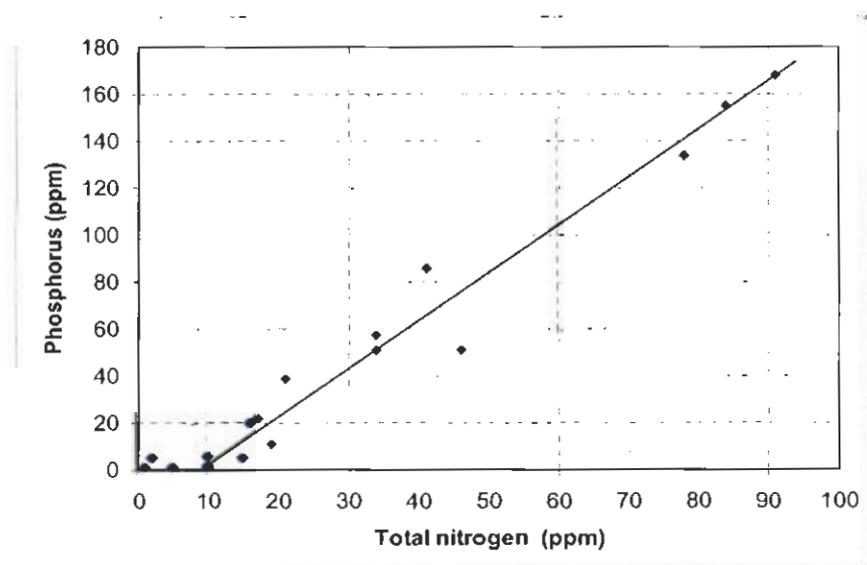
Due to the absence of fatty acid signal on NMR spectra, the presence of degradation products from lipids is strongly suspected. The loss of fatty acid chain of the LPC leads to the formation of two possible compounds, glycerophosphocholine and, with further degradation, phosphocholine. Such molecules are hardly extractible from glucose syrups and cannot be detected directly in WSHs with HPLC (e.g. ionic chromatography with conductimetric detection).

However, indirect analysis can be performed using the very accurate and sensitive phosphorus atomic absorption techniques. If glycerophosphocholine and phosphocholine are present in WSHs, there should be a strong correlation between nitrogen and phosphorus because those molecules carry one atom of N and one atom of P.

The *figure n°10* shows the relationship between nitrogen content and phosphorus content in AAC samples. It is noteworthy that the slope of the correlation curve is near 2 corresponding approximately to the molar mass ratio of Phosphorus/Nitrogen that is equal to 2.2. Thus, one mole of P corresponds to one mole of N.

Moreover, the “zero phosphorus” intercept is about 10 ppm of nitrogen. This suggests that  $\leq 10$  ppm of N does not come from lipid hydrolysis compounds but obviously from residual proteins or peptides. Therefore, the protein content can be estimated to be below 60 ppm. The range of residual protein in WSH shown by the P. Ferranti study is 5 - 45 ppm. **P. Ferranti's results and our observations seem to be in accordance.**

*Figure n° 10: Total Nitrogen vs Phosphorus (ppm)*



### III-3-3 Ethanol amine

Ethanolamine was detected on amino acid chromatograms and quantified (see chromatogram *figure n° 6*). Nitrogen from ethanolamine detected in WSHs ranges from <0.1 to 7 ppm (see *table n°4*). Ethanolamine nitrogen represents therefore nearly from 5 to 10% of total N in WSHs.

### III-4 Conclusion

WSHs have <5 to 100 ppm of total nitrogen analysed with Kjeldahl procedure.

HPLC amino acid determination shows that each sample contains less than 100 ppm of true proteins. **Therefore only a part of N is due to proteins (N<15 ppm).**

Direct amino acid analysis of WSHs with HPLC was found to be rather a robust procedure to quantify true protein content but the limit of detection is 100 ppm. Nevertheless further work to improve this detection limit would give us complementary information.

The most of nitrogen (up to 90%) in WSHs comes from lipids hydrolysis compounds, possibly glycerophosphocholine and phosphocholine. A small part of N is due to the presence of LPE or degradation compounds of LPE.

#### **IV Conclusion:**

- From an AAC survey on industrial wheat starches, the total nitrogen content ranges from 0.026 % to 0.058 %, the average value is 0.038 %. The major nitrogen compounds are lipids, (mainly LPC) which represent 60% of the total nitrogen. Principal proteins, 30% of total nitrogen in starch, are non-gluten proteins. The major protein is the GBSS, involved in biosynthesis of starch.  
Gluten represents only a small part of granule starch nitrogen, being less than 10 %.

- Nitrogen content of wheat starch based hydrolysates is <100 ppm.  
No wheat starch based hydrolysates have a (true) residual protein content more than 100 ppm (N<15 ppm). Up to now, HPLC total amino acid determination is the only method that allows to analyse true protein content in WSHs with a 100 ppm detection limit.

Lipid hydrolysis compounds are the principal nitrogen containing compounds and can explain up to 90% of total nitrogen. Glycerophosphocholine and phosphocholine presence is strongly suspected and would suggest that hydrolysis of lipids occurs in production and purification process.

From the quantification of the lipid hydrolysis compounds and total nitrogen, it may be deduced that residual proteins range from 0 to 60 ppm.

## References

- Acker L, Schmitz H, Wheat starch lipids 2. Identification and isolation of lysolecithin. *Starch* 19 ;233-239.
- Baldwin P, Starch Granule-associated proteins and polypeptides: a review. *Starch* 2001; 53: 475-503
- Denyer K, Edwards A, Martin C, Smith A.M, The mechanism of amylose synthesis: in *Starch : structure and functionality*, Ed P.J. Frazier, A.M. Donald and P. Richmond. 1996; 222 - 229
- Kluth A, Sprunck S, Becker D, Lorz H and Lutticke S. 5' deletion of a GBSS1 promoter region from wheat leads to changes in tissue and developmental specificities. *Plant Mol. Biol.* 2002; 49 (6): 669-682.
- Konieczny-Janda G. and Richter G. Progress in the enzymatic saccharification of wheat starch. *Starch* 1991, 43: 308-315
- Morrison W R. Lipids in cereal starches: a review. *J Cereal Sci* 1988; 8: 1-15
- Murai J, Taira T, Ohta D. Isolation and characterization of the four Waxy genes encoding the granule-bound starch synthase in tetraploid wheats *Appl. Biol. Sci.* 1999; 5: 31-42
- Skerritt JH, Hill AS. How 'free' is 'gluten free'? Relationship between Kjeldahl nitrogen values and gluten protein content for wheat starches. *Cereal Chem* 1992;69:110-2.
- Skerritt JH, Frend AJ, Robson LG, Greenwell P. Immunological homologies between wheat gluten and starch granule proteins. *Journal of Cereal Science* 1990a;12:123-136
- Sulaiman BD, Morrison WR. Proteins associated with the surface of wheat starch granules purified by centrifuging through caesium chloride. *J Cereal Sci* 1990;12:53-61
- Rahman S, Kosar-Hashemi B, Samuel MS, Hil A, David CA, Skerritt JH , Preiss J, Appels R, Morell MK The major proteins of wheat endosperm starch granules. *Aus. J. Plant Physiol.* 1995; 22: 793-803
- Vos Scheperkeuter Identification of granule-bound Starch synthase in potato tubers. *Plant Physiol.* 1986 ; 82 , 411-416.

## Annex 1: Descriptions of the methods

### Amino acid analysis (pr NF EN ISO 13-903)

The amino acids, released by hydrochloric hydrolysis, are adsorbed on a cation exchange column and eluted at 40°C by three buffers containing lithium, with an increasing pH and ionic strength.

The amino acids (A.A.) are detected at 440 to 570 nanometres after a post column reaction with ninhydrin.

### Total Nitrogen determination (NF EN ISO 5378)

Total nitrogen analysis is carried out following the Kjeldahl procedure: the sample is digested in boiling sulphuric acid in the presence of a catalyst. The nitrogen is converted to ammonium sulfate. When cool the digest is made alkaline and the ammonia distilled. The final step involves a final spectrophotometric determination of the ammonia using Nessler's reagent.

### Quaternary ammoniums by <sup>1</sup>H-NMR spectrometry

Quaternary ammoniums (expressed in ppm nitrogen) are determined with <sup>1</sup>H-FT-NMR spectrometry. Sample is dissolved into per-deuterated dimethylsulfoxide (DMSO-d-6). 3-(trimethylsilyl)-1-propane-sulfonic acid (TSPSA), as internal standard, and deuterated trifluoroacetic acid (TFA-d-1) are then added to the solution. Analysis is performed on a 250 MHz FT-NMR spectrometer, at 60 °C.

### Phosphorus analysis

The method used for the determination of Phosphorus in starch hydrolysis products is optical emission spectrometry (ICP-OES).

After dissolution in distilled water and addition of nitric acid, the measurements are performed by emission spectrometry.

The instrument is first calibrated with standard solutions at a specific emission wavelength of Phosphorus (213, 618 nm).

## Annex 2:

### Nitrogen content and total amino acid content in wheat starch

	Total nitrogen Kjeldahl method		Amino acids HPLC
	Nitrogen ppm	Nx6.25 ppm / cp (Apparent proteins)	Amino acids ppm / cp (true proteins)
Wheat starch n°1	272	1700	680
Wheat starch n°2	416	2600	690
Wheat starch n°3	336	2100	660
Wheat starch n°4	265	1655	640

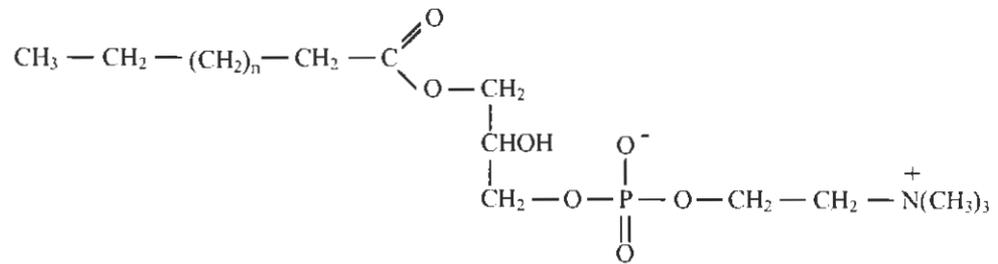
### Amino acid composition of residual protein in wheat starch

Amino acids (mg/kg = ppm / cp)	Wheat starch n°1	Wheat starch n°2	Wheat starch n°3	Wheat starch n°4
Aspartic Acid	75	72	72	72
Threonine	30	29	25	27
Serine	43	35	37	32
Glutamic Acid	93	85	95	84
Glycine	49	44	47	43
Alanine	46	40	45	43
Valine	57	56	57	55
Isoleucine	30	29	33	29
Leucine	58	56	63	57
Tyrosine	15	17	15	15
Phenylalanine	31	33	35	33
Lysine	46	47	47	46
Histidine	24	21	31	18
Arginine	51	53	51	56
Proline	31	44	35	30
Total	679	661	688	640

Mean value of **true protein** from 5 wheat starches = 667 ppm /cp

## Annex n°3

### LPC : Lysophosphatidylcholine



### LPE : Lysophosphatidylethanolamine

