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DEVELOPMENT OF AN INTEGRATED STRATEGY FOR CONTROLLING THE ALLERGEN ISSUE IN THE BELGIAN FOOD AND CATERING INDUSTRY

"ALLERRISK"

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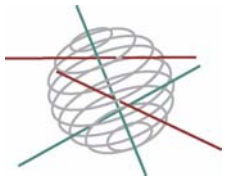

Agri-food

FINAL REPORT PHASE 1

DEVELOPMENT OF AN INTEGRATED STRATEGY FOR CONTROLLING THE ALLERGEN ISSUE IN THE BELGIAN FOOD AND CATERING INDUSTRY

"ALLERRISK"

SD/AF/03A

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1. Summary Project

Food allergies represent an important health problem and the prevalence of allergic reactions shows an increasing trend over the last years. European and American studies showed that food allergies affect up to 2 % of the adult population and up to 8 % of the children. Currently, the only effective treatment for food allergy is avoidance of the allergen-containing food. However, total avoidance is sometimes difficult since processed food products may contain a broad variety of ingredients, of which some have the potential to induce allergenic reactions. Sensitive individuals may also be inadvertently exposed to allergenic proteins by consumption of food products that are supposed to be free of a certain allergen. Food products may contain impurities caused by carry-over due to inadequate cleaning of shared processing equipment (e.g. during transport, storage and processing) or through rework of allergen containing products. The food industry is, due to the lack of an integrated approach for the detection of allergens, confronted with a problem with regard to the validation of their production processes in order to assure absence of cross-contamination in the production lines and also with a reliable quality control of the incoming materials.

Reliable detection and quantification methods for food allergens are necessary in order to ensure compliance with food labelling and to improve consumer protection. However, the detection of allergens in food is not easy as they are often present only in trace amounts or masked by the food matrix. The methods used are either targeting the allergen (protein) itself or a marker that indicates the presence of the offending food. As markers for the detection of potentially allergenic food products or ingredients, specific proteins or DNA fragments are targeted. ELISA and PCR methods are the methods of choice for developing routine methods. They both have their characteristic merits and drawbacks concerning their applicability in the detection and quantification of allergens in foodstuffs. It is clear that there are still a lot of problems in the detection of allergens that have to be cleared out. An integrated strategy to control the allergen issue in Belgium is lacking, which is a serious problem for the industry, the government and most important the allergic patients. Therefore the main objective of this project is to develop for selected allergens such an integrated strategy. In such a strategy, quantitative analysis of the target allergen plays a key role. The allergens selected for this project are soy and hazelnut. This selection was based on a number of criteria, including the likelihood of the presence of these hidden allergens due to cross contamination in foods, the severity of the allergic reaction, the number of patients confronted with such an allergy and the number of available serum samples present in the serum collection of the University hospital of Antwerp.

Commercially available screening tests for detection of hazelnut and soy (PCR) and allergenic proteins thereof (ELISA) will be validated. The robustness of the available methods to detect soy and hazelnut proteins in foodstuffs will be evaluated. Finally the effect of different processing steps on the detection and the

allergenic reaction will be studied. Therefore a stepwise approach for simulating the influence of food processing on the allergen is proposed in which at a first stage the impact of chemical reactions on protein and DNA level are studied in simple aqueous solutions. In a second stage, similar chemical reactions will be monitored in a food model system. The impact of these food processing simulating reactions on the chemical composition of the allergen, on its detection by commercially available methods and on its allergenicity will be monitored using the following 5 analytical approaches: immunochemical analysis; PCR analysis; mass spectrometric analysis; chemical analysis and *in vitro* allergenicity assessment.

New integrated analytical methods will also be developed and optimized. It is expected that different food processing steps will decrease the robustness of the existing immunological and DNA based methods. It is the aim to detect correlations between the results of the different analytical strategies and the allergenic capacity of a food product. The goal is to define the different parameters that have an impact on this correlation and where possible to quantify them. For those proteins isolated from the target allergic agricultural raw materials not showing loss of allergenicity during food processing and showing loss of detectability by commercially available routine methodology new ELISA's and PCR's will be developed.

The results obtained with screening methods have to be confirmed by mass spectrometric methods. Therefore, the applicability of a quantitative technique of analysis based on liquid chromatography coupled to mass spectrometry will be tested on the allergens. This approach is standard practise in proteomics

The obtained detection limits of the commercially available methods and the newly developed methods (ELISA,PCR, MS) will be evaluated by in-vitro techniques. In this way information will be available how far these detection limits can give 100 % guarantee to the allergic patient and consumer in general.

This project also aims to provide practical guidelines for reducing allergenic risks in food industry and in the catering. Contaminations can lead to allergic reactions in sensible patients. The guidelines are of interest for the autocontrol HACCP-based systems, which are installed and controlled by the Belgian Food Safety Agency (FAVV).

Due to the lack of an integrated approach for the detection of allergens the food industry is at the moment confronted with a problem with regard to the validation of the production processes for the absence of allergenic compounds in the final products. Critical points are cross-contamination in their production lines and quality assurance of the incoming materials. Rework of allergen-containing products can also be a source of allergens in the final food products. In the last part of this project it is proposed to use the developed methods in Belgian food factories belonging to the dairy sector, chocolate sector, meat sector and cookie sector to evaluate the cleaning processes applied and to assess the critical control points in the quality management system. Besides the food industry, different kitchens including catering, are confronted with the problem of controlling cross-contamination to avoid allergenic reactions of the consumers. Autocontrol systems, applied in catering are necessary for the most important allergens to assure a

proper communication to the clients. Because in a hotel school mini-installations are available, similar to those used in catering companies, the same general approach as for the food industry will be evaluated under different conditions. As this is the place where people are educated to work in the catering industry the outcome will have a secondary effect at the level of sensitising.

2. Introduction

2.1. Context

The last few years the incidence of food allergies has increased; about 6-8% of children and 2-3% of adults suffer from a food allergy (Ortolani et al., 2001). According to the World Health Organisation food allergies pose the fourth most important public health problem. About 15-20% of the population is (in)directly confronted with food allergies in their daily life through a family member, at work, at school, etc.. Currently, the only effective treatment for food allergy is total avoidance of the allergen-containing food. This is not always feasible as food products often are composed of a large variety of ingredients of which some could cause an allergic reaction. Food products can also unintentionally contain allergenic components due to cross-contamination during storage or shipment or as a result of rework or inadequate cleaning.

Directive 2003/89/EC, amending Directive 2000/12/EC, contains a list of allergenic foods, which have to be declared unambiguously in the list of ingredients on the package label. The lack of an integrated approach for the detection of (food) allergens puts the food industry for a problem with regard to the control of their production processes to ensure the absence of cross-contamination. There is an urgent need for validated detection and quantification methods which are specific, sensitive, reproducible and compatible with different matrices. The currently available methods detect either the allergenic proteins (ELISA) or a DNA marker of the allergenic component/product (PCR). In addition, mass-spectrometric methods are being developed to quantify allergens.

2.2. Objectives

The objective of this project is to develop an integrated control strategy for 2 selected allergenic foods, namely soy and hazelnut.

The performance of the currently commercially available detection kits will be validated for their robustness. It is expected that different food processing steps will have an influence on the proteins and their structure. This can have an effect on the detection but does not necessarily imply that the same is true for the allergic effect they elicit in a patient. To monitor the effect of food processing on the allergens, different chemical changes will be induced. In the first part the effect of the chemical reactions will be studied in an aqueous buffer, in the second part the same reactions will be performed in a model food system. The impact of these chemical changes on the detection of the allergens will be approached by ELISA, PCR, chemical analysis, mass spectrometry, immunoblot and *in vitro* functional

allergy tests. The correlation of the results with the different analysis methods will be evaluated. It is expected that some reactions will have an effect on the detection without altering the allergenicity of the proteins. It is the aim to define those reactions and to develop new ELISA and PCR methods to detect those proteins, which do not lose their allergenicity after food processing.

As a confirmation method for the above mentioned screening methods a mass spectrometric method will be developed. The applicability of a quantitative technique of analysis based on liquid chromatography coupled to mass spectrometry will be tested on the allergens.

The obtained detection limits of the commercial kits and the newly developed methods will be evaluated with *in vitro* allergenicity tests. In this way information will be available on how far these detection limits can give 100 % guarantee to the allergic patient and the consumer in general.

The final goal of this project is to provide practical guidelines for reducing the allergenic risks in the food industry and the catering. This part of the project will be done in co-operation with different factories of the food industry and a hotel school where mini-catering installations are available. Both are confronted with a problem with regard to the validation of the production processes for the absence of allergenic compounds in the final products. Different critical control points (CCPs) will be defined with regard to cross-contamination of their products with allergen-containing foodstuffs. Cross-contamination can occur at the level of the raw materials during shipment or storage, at the level of the production process or at the level of the cleaning process. The defined CCP's will be tested in practice to evaluate their relevance and finally preventive measures and control limits for the CCPs will be defined. These guidelines will be formulated to the Belgian Food Safety Agency (FAVV) as a proposal for implementation in the autocontrol guides that are used to set up a HACCP system.

2.3. Expected outcomes

This project will result in an integrated approach for soy and hazelnut allergens, which could be applied in the future to other allergens. The project aims thus at providing the government and producers with tools to control the legislation concerning allergens in a reliable way. The screening of food products (and their production and processing sites) to detect possible allergens is an essential part of a solid preventive policy. The results of work package one will on the one hand give an overview of the performance characteristics and robustness of the currently commercially available kits for detection of hazelnut and soy in food products. On the other hand the profound study of the controlled chemical reactions on the hazelnut/soy proteins will give insight in the behaviour of proteins during food processing. With the newly developed real-time PCR in work package two, the objective is to detect to detect hazelnut after food processing, targeting one or more genes coding for hazelnut allergens. Work package three will provide a mass

spectrometric confirmation method for the screening methods, such as ELISA and PCR.

2.4. Research team

The mission of ILVO consists of the execution and coordination of policy-supporting scientific research and its associated services, with a view to sustainable agriculture and fisheries in relation to economical, ecological and social perspectives. Emphasis is laid on food safety items in the research group dealing with this project.

The Unit Food Chemistry and Human Nutrition focuses her research on the chemical behaviour of foods and food ingredients and the impact of this behaviour on human health. The research of the Laboratory of Protein Biochemistry and Protein Engineering is dedicated to the mass spectrometric analysis of proteins and peptides and to proteomics.

In the Centre of Analysis of Residues in Traces, the fundamental laboratory research is dedicated to molecular recognition. The behaviour of biological molecules is studied both theoretically and experimentally and the most important tool in the laboratory is the mass spectrometer.

The Department of Immunology, Allergology and Rheumatology focuses on the development of new diagnostic tools which can be helpful in the assessment of allergenicity of native and recombinant allergens.

3. Results

Work package 1: Evaluation of the robustness of the available methods

3.1.1. Functional evaluation of different protein extraction procedures

- Partners

  ILVO, UA

- Objective

This study aims to evaluate existing commercially available methods and develop new methods to detect food allergens. These methods are needed for the industry and catering to control their production processes to be able to protect allergic consumers. In this kind of study it is important from a clinical point of view to use an allergen extract in which the allergenic properties, i.e. the capacity to elicit an allergic response, are conserved. However, there is no standard protein extraction procedure available for allergen-containing foods. The literature describes many different protocols, most of them applying an aqueous protein extraction. Some of them make use of protease inhibitors (Bjorksten et al., 1980; Bolhaar et al., 2004; Kopper et al., 2005; Rudeschko et al., 1995), while others not. Defatting hazelnuts with hexane (Dooper et al., 2008; Yeung and Collins, 1996), acetone (Beyer et al., 2002) or diethyl ether (Enrique et al., 2005) before protein extraction is sometimes described as hazelnuts have a fat content of approximately 60%. Watanabe et al. (Watanabe et al., 2005) described a method for extracting proteins from processed foods, in which the buffer contains SDS, a surfactant, and 2-mercaptoethanol, a reducing agent.

This task aims to compare the use of protease inhibitors during extraction and defatting hazelnuts/soybeans before extraction.

- Methodology

The use of protease inhibitors was investigated applying 2 protocols, previously described for the extraction of proteins from allergen-containing foods; method A (Kujala et al., 2002) is a classical extraction with PBS, similar to method B (Bjorksten et al., 1980), however, the latter describes the use of protease inhibitors. To study the possible influence of defatting hazelnuts before extraction a third protocol was selected, including a hexane defatting step (Yeung and Collins, 1996).

Table 1: Overview of the different extraction protocols

Extraction buffer		Enzyme inhibitors
Extract A (Kujala et al., 2002)	PBS, pH 7.4	2mM EDTA 5mM DETC (diethyl-dithiocarbamate) 0.5mM BAHC (benzamidinehydrochloride) 0.2mM PMSF (polymethylsulphonefluoride)
Extract B (Bjorksten et al., 1980)	PBS, pH 7.4, 2% PVPP * not included in dialysis buffer	
Extract C (Yeung and Collins, 1996)	20mM NaH_2PO_4, 1M NaCl pH 7.4	
Extraction procedure	Defatting step only for extract C: - 10g material + 10ml hexane - shake 30min at RT - centrifugation, 5min, 2000g - take of supernatant - repeat 3 times - dry ON at RT	Extraction: - (equivalent of)10g material + 100ml extraction buffer - shake ON at 4°C - centrifugation, 1h, 40.000g - dialyse against extraction buffer, ON, 4°C - lyophilisation - storage at –20°C

Nine different brands of hazelnuts were purchased in Belgian local supermarkets, which represent what is consumed in Belgium. One of the products concerned roasted hazelnuts, as hazelnuts are often roasted before processing in food products. Two Turkish qualities of hazelnuts (Levant and Giresun) were also available. As Turkey is the world leader in hazelnut export, these are good representatives for hazelnuts in food products. A mix of the hazelnuts was made by taking one kg of each kind. Soybeans were provided by Alpro (Belgium) and Cargill (Belgium). Again, a mix was made of the different soybeans.

Hazelnuts/soybeans were frozen with liquid nitrogen and ground in a two-step process first with a Moulinex blender and secondly with an Ultra Turrax t25.

Evaluation of the different extracts was performed by IgE-immunoblot and the Basophil Activation Test (BAT). The Basophil Activation Test (BAT) is a flow-cytometric analysis of *in vitro*-activated peripheral blood basophils, which rests upon quantification of changes in expression of basophilic activation markers after challenge with a specific allergen (Ebo et al., 2006). The activation marker used in this study is CD63, whose expression on the outer surface membrane is upregulated in IgE-activated basophils. Basophils are activated by cross-linking of IgE's through an allergen. Basophils are characterised with an anti-IgE antibody and the activation status is analysed with an anti-CD63 antibody.

o Results

For the investigation of the use of protease inhibitors, four hazelnut extracts were made: extract A+ and B+, including the mix of protease inhibitors as

described for method B, and extract A- and B-, excluding the inhibitors. These extracts were analysed by IgE-immunoblot with blood from four hazelnut-allergic patients and one control patient (hazelnut tolerant). Figure 1 shows that not including the mix of protease inhibitors resulted in a lot of aspecific staining, which is an indication for the presence of degraded proteins.

In the BAT the different hazelnut extracts were analysed at different concentrations, which resulted in dose-response curves (1ng/ml – 1mg/ml) (Figure 2). The results of the BAT confirmed those from the immunoblot test, i.e. that the quality/allergen-provoking potential of the extracts prepared without protease inhibitors is decreased. Figure 2 shows that with extract A- a smaller percentage of activated (CD63+) basophils is measured for 1 patient (red curve) and with extract B- for 2 patients (red and black curve).

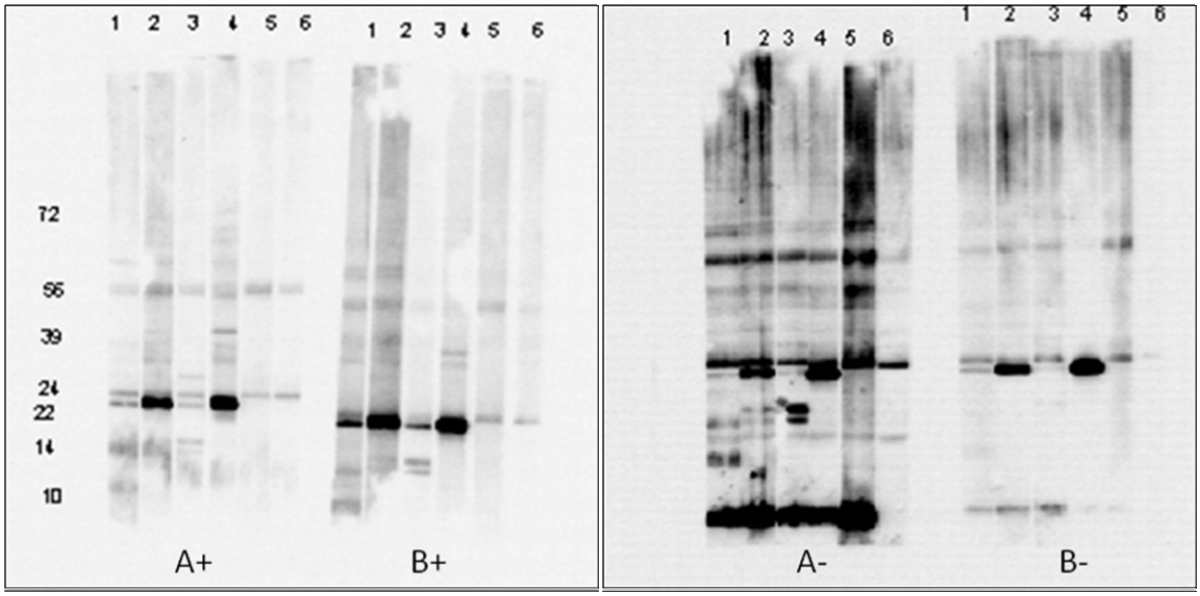


Figure 1: IgE-immunoblot of hazelnut extracts, prepared with (A+, B+) or without (A-, B-) protease inhibitors, with serum from four hazelnut-allergic patients (lane 1-4), one control patient (lane 5) and a blanc (lane 6)

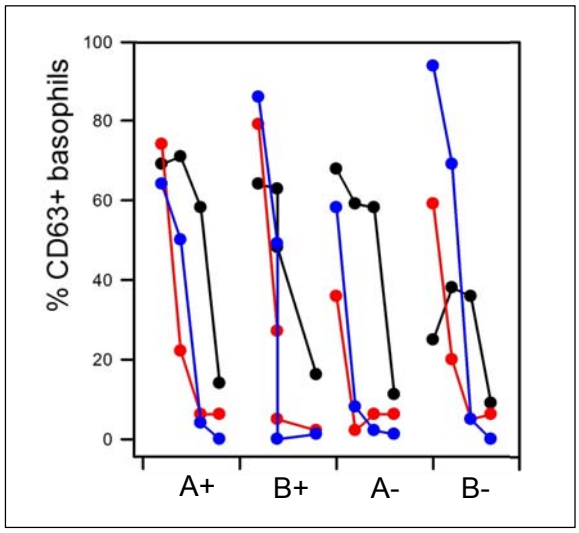


Figure 2: Dose-response curve in BAT of extract A and B prepared with (+) or without (-) protease inhibitors with blood from three hazelnut-allergic patients (blue, red and black curve)

To study the possible influence of defatting on the allergenicity extracts A, B and C were prepared with inclusion of the mix of protease inhibitors, as this had proven to improve the quality of extracts, and analysed with serum from two hazelnut-allergic patients and one control patient. Distinct proteins were recognised with the serum from the hazelnut-allergic patients, while no binding was seen for the control patient.

The IgE's from the two patients bound to different proteins, however, each patient recognised the same proteins in the different extracts, indicating the same allergens are present in the three extracts.

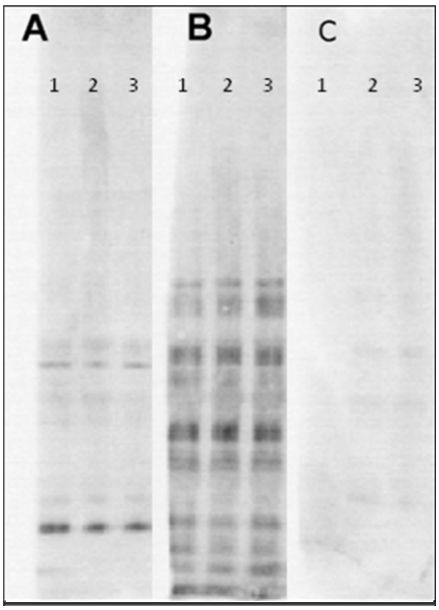


Figure 3: IgE-immunoblot of different hazelnut protein extracts (1=extract A, 2=extract B, 3=extract C) with serum of two hazelnut-allergic patients (A,B) and one hazelnut-tolerant patient (C)

The allergenicity of the different extracts was tested by the BAT with blood from three hazelnut-allergic patients (Figure 4). The response differed between patients, but each individual patient showed the same response to the three extracts. For these patients defatting does not influence the allergenicity of the extract.

The same test was performed with analogous extracts made from soybeans with serum from three soy-allergic patients (Figure 5). The basophils of two out of three patients had a similar response after stimulation with each extract.

- Conclusion

The results from Figure 1 and Figure 2 show that the use of protease inhibitors is essential for the stability of the proteins and consequently the allergenicity of the allergen extract. Therefore, for all future tests protein extracts will be prepared with the inclusion of the mix of protease inhibitors, as described aboveTable 1.

Figure 4 and Figure 5 indicate that the three protocols produce an equally allergenic protein extract. Moreover, defatting is unlikely to influence the quality of the extract, for hazelnut as well as for soy.

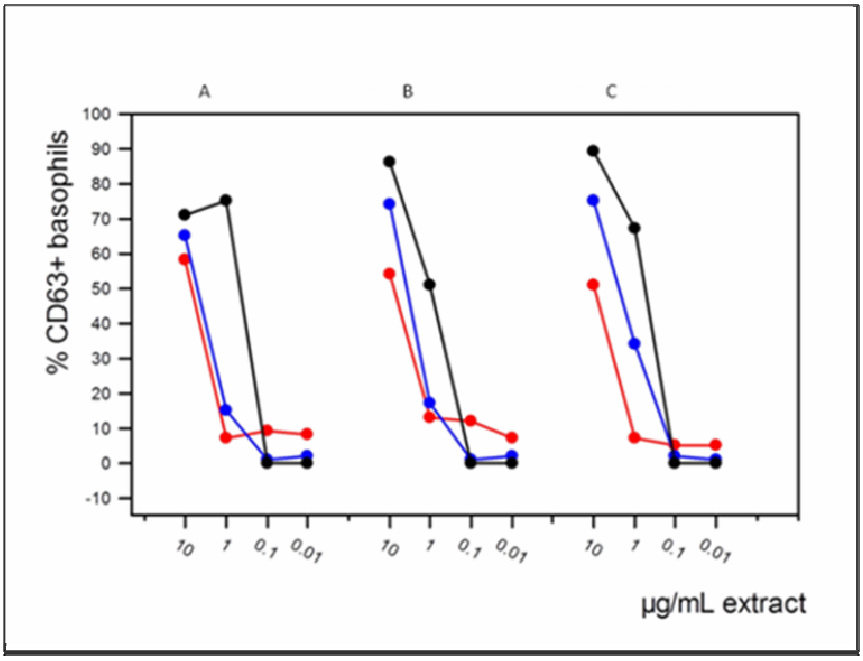


Figure 4: Dose-response curve in BAT of the different hazelnut protein extracts (A, B, C) prepared with protease inhibitors with blood from three hazelnut-allergic patients (blue, red and black curve)

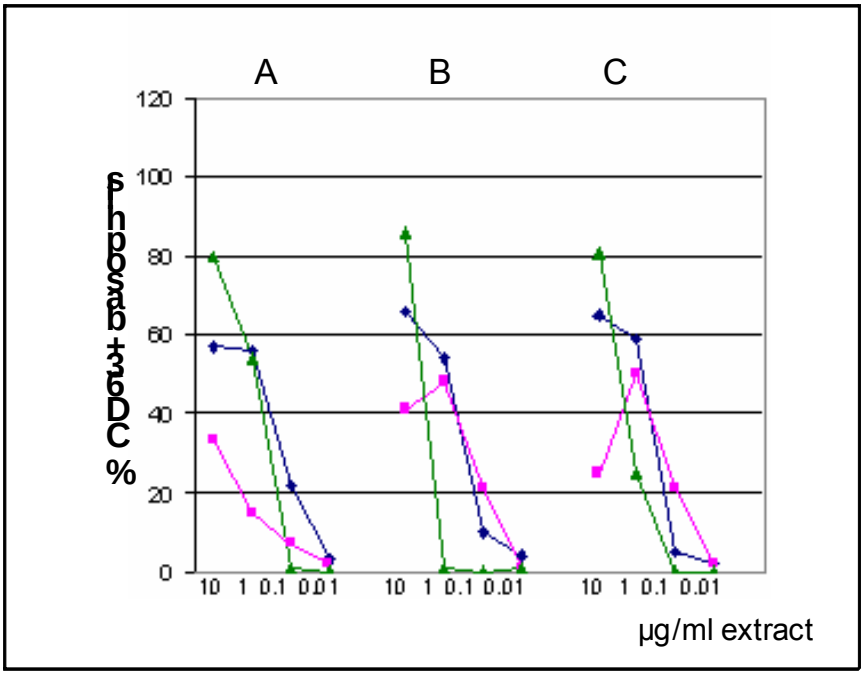


Figure 5: Dose-response curve in BAT of the different soy protein extracts (A, B, C) prepared with protease inhibitors with blood from three hazelnut-allergic patients (blue, green and pink curve)

3.1.2. Validation of commercially available ELISA kits as such and after chemical treatment

- Partner

  ILVO

- Objective

Different parameters will be determined to evaluate the performance of the different commercially available ELISA kits to detect soy and hazelnut in food products. Therefore, pure protein extract will be used on the one hand, prepared according to method A (see above) with protease inhibitors. On the other hand, spiked matrix will be used to determine possible matrix effects. Food processing affects proteins in different ways. The impact thereof on the detection of proteins/allergens will be tested by applying specific chemical reactions to the proteins which mimic food processing (see 2.1.4).

A critical point when preparing a spiked matrix is the homogeneity of the material; it is of utmost importance that the spiked analyte is spread evenly through the matrix. Two recipes were tested for the preparation of cookies spikes with hazelnut.

- Methodology

An inventory was made from all the currently commercially available ELISA kits for the detection of hazelnut and soy in food products (Table 2).

Table 2: Commercially available ELISA kits for hazelnut and soy

	Kit	Manufacturer	type
HAZELNUT	Ridascreen Fast Hazelnut	R-Biopharm	s-ELISA
	Biokits Hazelnut assay kit	Tepnel Biosystems	s-ELISA
	Hazelnut Residue assay	ELISA Systems	s-ELISA
	Veratox for Hazelnut	Abkem Iberia	s-ELISA
	Hazelnut Diagnokit	Neogen	Indirect competitive ELISA
SOY	Soy Residue Immunoassay	Safe Path	s-ELISA
	Soy Protein Residue ELISA	ELISA Systems	s-ELISA
	Veratox for Soy	Neogen	s-ELISA

For each kit a standard curve was first set up by calculating the best fitting curve for the OD-values of the standards included in the kit with following equation:

$$OD = \left[\frac{(b-a)}{\left(1+\frac{conc}{c}\right)\wedge d} \right] + a$$

In which
a = maximum OD-value
b = minimum OD-value
c =E_{50} (concentration at half-maximal saturation)
d =slope
conc = concentration of the standard/sample

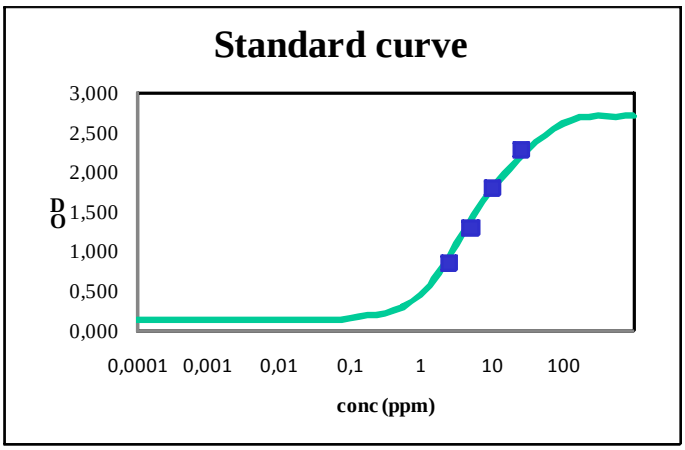


Figure 6: Example of a standard curve obtained with the standards of the Veratox for Hazelnut kit

Application of the chemical reactions was performed as described under 2.1.4.

Spiked matrix (cookies fortified with hazelnut) was prepared according to the recipes described by Poms *et al. (Poms et al., 2005)* and Scaravelli *et al. (Scaravelli et al., 2008).* In the first recipe the dough is spiked before baking whereas in the second one the butter to prepare the dough is spiked. After baking the cookies were ground and the homogeneity of the material was checked by analysing the spiked material with the Veratox for Hazelnut kit.

o Results

Validation was first performed on the hazelnut kits with pure protein extract. Validation with spiked matrix and of the soy kits will be executed in the second phase.

Preliminary tests with the Hazelnut Diagnokit were unsatisfactory and did not fulfil the performance criteria indicated by the manual. The same problems were noticed with a second batch; therefore it was decided to exclude this kit from the study.

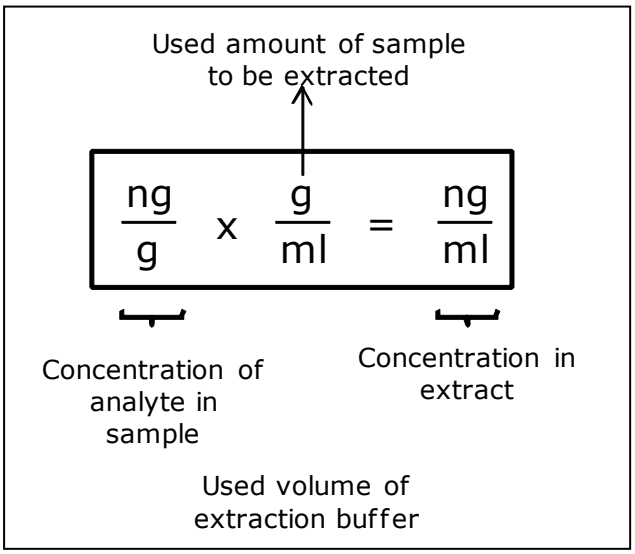
- ***LOD / LOQ***

The Limit of Detection was determined by analysing 20 blank samples, calculating the mean value and adding three times the standard deviation. For the Limit of Quantification six times the standard deviation was added. A blank sample is in this case the buffer in which food samples are extracted and/or diluted before analysis in the ELISA assay according to the manufacturer's protocol.

Table 3: LOD and LOQ as certified by the manual and determined with protein extract for the different hazelnut kits

	Veratox for HN	Ridascreen FAST	HN Residue Detection kit	Biokits HN Assay
LOD	-	1.5 ppm	-	0.1 ppm
$\overline{X}$ **+3*SD**	0.058 ppm	0.014 ppm	0.0003 ppm	0.023 ppm
LOQ	2.5 ppm	2.5 ppm	0.5 ppm	1 ppm
$\overline{X}$ **+6*SD**	0.123 ppm	0.044 ppm	0.0013 ppm	0.068 ppm

The LOD was also determined by deducing the minimum value of the standard curve (b-value) estimated by the fitting program and by determination of the concentration at which 95% of the samples are positive. In the latter case the protein concentration of the samples is known as expressed in ng/ml. When converting the determined concentration to ppm (=µg/kg), the resulting values were not in agreement with the LOD values determined by the other 2 approaches. The problem is that the kits are not clear in how the results are expressed. The concentration of the standards in a kit is mostly expressed in ppm. When converting this concentration to one in ng/ml, using the following equation, it is not clear if the 'ng' indicates hazelnut/soybean or hazelnut/soybean protein. This is however a very important issue to be able to interprete results and be able to compare different kits with each other.



- ***Specificity***

For the results of an ELISA test to be reliable it is important that the test is specific for the analyte to be detected. *Cor a 1*, one of the major hazelnut allergens belongs to the *Bet v 1*-related proteins. *Bet v 1* is one of the major allergens from birch pollen, consequently, hazelnuts and birch pollen can cross-react with each other. Cross-reaction can occur due to related protein structures or amino acid sequences. It is impossible to test all possible allergenic foods to determine the

specificity of a kit; therefore, an alignment was performed of the amino acid sequence of all known hazelnut and soy allergens with BLAST. This resulted in a list of proteins with a certain percentage of sequence similarity, of which the origin was determined. The most frequent sources were selected to be tested in the different ELISA kits.

Another way to approach specificity is to test the bulk ingredients present in food products. These ingredients are present in very high levels and are more likely to cause false-positive results due to cross-reaction. This study will mainly focus on cookies; therefore the main ingredients of common cookies will also be tested in the specificity analysis.

To test the specificity of the hazelnut kits, the tree nuts and peanut mentioned in Directive 2003/89/EU will also be included.

Testing will be performed with a pure protein extract of the selected products.

Table 4: Selected foods to test the specificity of the ELISA kits

	HAZELNUT	**SOY**
Bulk proteins	milk, egg, wheat, maize, rice, soy, rye, barley	milk, egg, wheat, maize, rice, soy, rye, barley
Cross-reacting proteins	grape, pea, fig, coffee, coleseed, linseed	grape, pea, beans, peanut
Nuts	pecan, macademia, walnut, almond, cashew, paranut, pistache, peanut	

- ***Influence of chemical modification on detection***

Maillard reaction

Maillard reaction was performed on hazelnut protein extract in the presence or absence of glucose for 48h (details see 2.1.4). Samples were taken at time 0 and after 24 and 48 hours. The sample at time 0 is the reference sample as it did not undergo Maillard reaction. A dilution series was made in the extraction and/or dilution buffer of each ELISA kit. Maillard reaction performed in the absence of glucose did induce no or a small reduction in detection of the hazelnut proteins in the different kits (Figure 7). In the presence of glucose no reduction was seen with the Ridascreen FAST Hazelnut kit. In the other kits the detection was reduced. With the Veratox for Hazelnut and the Hazelnut Residue Detection kit detection was decreased more after 48h than after 24h. With the Biokits Hazelnut Assay a decrease was seen only after 48h.

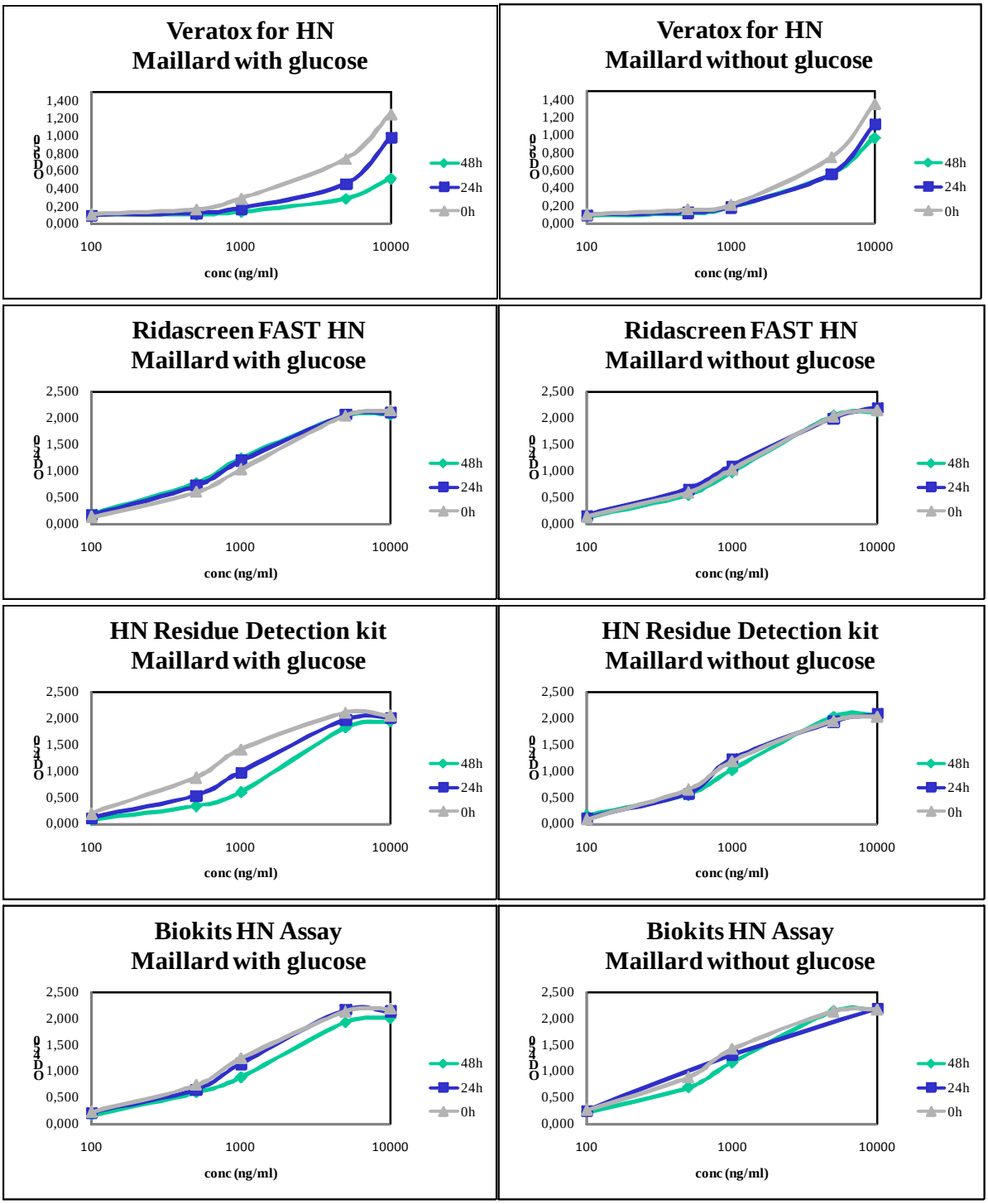


Figure 7: Analysis of hazelnut protein extract after Maillard reaction in the presence of absence of glucose in the different ELISA kits

Oxidation

Hazelnut protein extract was oxidised with HOCl at pH 8 and pH 5.8 (details see 2.1.4). The analysis was not performed with the Hazelnut Residue Detection kit as it was out of stock at the moment.

No difference is seen between oxidation at pH 5.8 and pH 8 (Figure 8). Oxidised samples could not be detected any more with the Veratox for Hazelnut kit. With the Ridascreen FAST Hazelnut and the Biokits Hazelnut Assay the detection of the strong oxidised samples (5mM HOCl) was lower than the less oxidised ones (2.5mM HOCl).

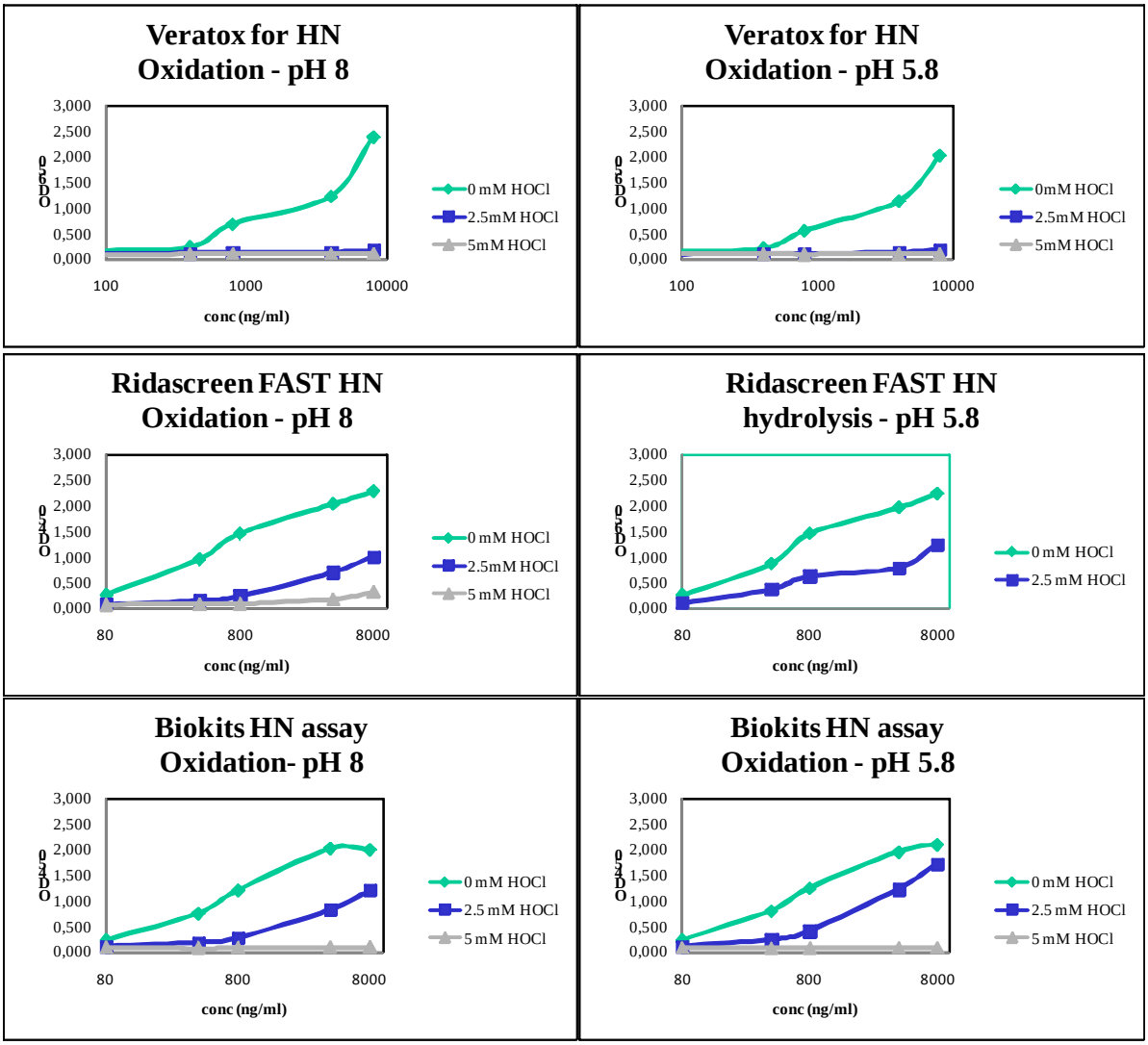


Figure 8: Analysis of hazelnut protein extract after oxidation with HOCl (2.5 and 5 mM) at pH 5.8 and 8 in the different ELISA kits

Hydrolysis

Pepsin-hydrolysed hazelnut protein extract (see 2.1.4) was for the moment only analysed with the Veratox for Hazelnut kit. Samples hydrolysed for 15min or 30min showed the same degree of reduced detection (Figure 9).

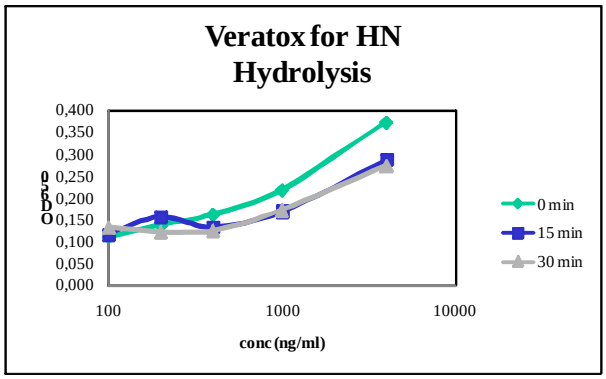


Figure 9:Analysis of hazelnut protein extract after hydrolysis with pepsin in the different ELISA kits

- ***Spiked matrix: homogeneity testing***

First, cookies were made according to the recipe described by Poms *et al.* in which the dough is spiked with ground hazelnut. Cookies containing 5ppm and 10ppm hazelnut were analysed for their homogeneity. Ten replicates were extracted and analysed *in duplo* with the Veratox for Hazelnut kit according the manufacturer's protocol.

From Table 5 it can be concluded that the hazelnut was not spread homogeneously through the dough. For the cookies spiked at 5 ppm one sample has a concentration much higher than the concentration of the other samples, which have a very low concentration. The same is true for four samples of the cookies spiked at 10ppm.

Table 5: Analysis of homogeneity of spiked cookies prepared according to Poms *et al.*

Spiked level	Retrieved concentration									
5 ppm	0.40	0.26	0.37	0.30	0.26	4.56	0.56	0.39	0.38	0.54
10 ppm	0.90	20.21	1.45	1.33	6.07	1.35	1.24	3.81	27.84	1.16

Cookies were then prepared according to the second recipe described by Scaravelli *et al.*. On the one hand three replicates (1-3) were analysed in duplo, to check the homogeneity of the cookies. On the other hand the homogeneity of the butter was checked by analysing another three samples (4-6), obtained by taking 20 samples of the material and mixing it thoroughly again.

Table 6: Analysis of spiked cookies prepared according to Scaravelli *et al*

Spiked Level	Retrieved concentration					
	1	2	3	4	5	6
1 ppm	0.39	0.44	0.32	0.68	1.80	0.60
10 ppm	4.22	3.90	3.93	5.00	4.26	4.60

With this recipe the hazelnut seems to be homogeneously spread, except for some minor deviations at the 1ppm level.

- Conclusion

The LOD/LOQ values determined for the different kits are very low compared to the values provided by the manual of the kit. These measurements were done with pure hazelnut protein extract. Determination of the LOD/LOQ with matrix will better reflect the real situation and will therefore also be performed in a later stage.

To test the specificity of the commercial kits, a selection of bulk ingredients present in cookies was made, together with a list of sources containing proteins with similar amino acid sequences as the known allergens of hazelnut and soy.

After chemical modification the measured protein concentration differs depending on the kit used. This will probably depend on the nature of the antibodies used to construct the ELISA, determining what protein(s) is/are detected. However, this information is not available. It seems that inducing Maillard reaction has a more profound effect on the structure and consequently on the recognition of the proteins by the antibodies than oxidation. To make a conclusion concerning hydrolysis the other kits will have to be tested first.

As it is very important to produce a homogeneously spiked matrix to validate the different kits; the recipe according to Scaravelli et al. will be used to prepare cookies fortified with hazelnut/soybeans. Butter can be melted, making it easier to mix it with the ground hazelnuts/soybeans in a homogeneous way.

3.1.3. Optimisation of DNA extraction

- Partner

ILVO

- Objective

To optimise the yield of the DNA extraction, different pre-treatments of the starting material were tested. These were performed on hazelnut and peanut.

- Methodology

The raw material was frozen at –20°C before applying the pre-treatment. Material was either milled with an analytical mill (Retsch ZM-100 or Retsch ZM-200) or with a kitchen robot. The pre-treatments consisted of soaking the material overnight in water and/or freezing with liquid nitrogen before milling and extraction. DNA was extracted according to the Qiagen Plant DNEasy Mini kit (Westburg), manually or with the QiaCube Robot. The DNA concentration of the extracts was measured using a NanoDrop^{R} ND-1000 UV-VIS spectrophotometer (adsorption spectrophotometry). The integrity of the DNA was checked by loading the DNA extracts on a 1.5% agarose gel.

o Results

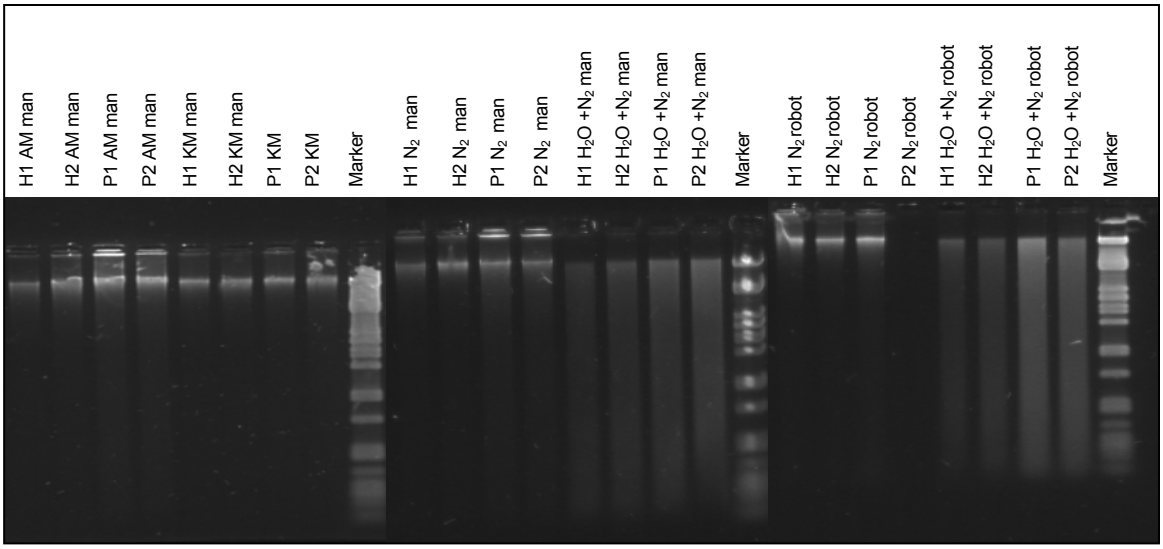


Figure 10: Analysis of hazelnut and peanut DNA extracts after pre-treatment (AM=analytical milling, KM=kitchen milling, N_2=freezing with liquid nitrogen, H_2O=soaking in water, man=manual extraction, robot=extraction with Qiagen robot)

The extraction yield was higher with analytical milling than with kitchen robot milling. Pre-treatment of the samples by soaking in water resulted in degraded DNA (Figure 10, smear on gel). The highest yield was obtained by pre-treatment with liquid nitrogen and analytical milling and in addition this resulted in non-degraded DNA.

The quality of the extracted DNA was further tested through PCR reactions targeting conventional sequences, such as 18S ribosomal DNA (different primers), an intergenic spacer in 18S ribosomal DNA (ITS1/2 primer pairs) and a non-coding chloroplast DNA-specific sequence (primers PLANT1/2). All extracts were amplifiable, which indicates that the DNA is of good quality and no PCR-inhibitors are present.

o Conclusion

The best way to treat samples before extracting DNA is freezing them with liquid nitrogen and milling them with an analytical mill. Interfering compounds, such as polysaccharides, RNA, proteins, fat fractions and salts have to be eliminated from the DNA extract. The Qiagen Plant DNEasy mini kit produces amplifiable and thus good quality DNA. Extraction with the Qiagen robot delivered some problems with regard to the presence of a fat layer, as nuts have a high fat content. Therefore, manual extraction is preferred.

3.1.4. Application of controlled chemical reactions to allergen proteins

o Partner

UGent

o Objective

Many foods are processed for various reasons: to increase food quality, improve taste and flavour and extend shelf life by inactivation of microbes and toxins. However, many of these processing steps have a profound influence on protein structure and hence on allergenicity and antigenicity (e.g., detectability by the immunological tests). Processing can elicit an unintentional effect on allergenic foods, but it may also be a tool to produce non-allergenic or hypo-allergenic foods (Poms and Anklam, 2004). Therefore, the aim of this study is to determine the influence of processing on allergenicity and detectability by the commercial available methods. The soybean and hazelnut allergen proteins are subjected to several controlled chemical reactions (in a buffer solution or in a suitable food model system) and their impact on the protein itself and on its immunochemical detection is assessed.

o Methodology

- ***Model systems***

Protein extraction

Hazelnut and soybean protein extracts were obtained according to the protocol described earlier by Yeung and Collings (Yeung and Collins, 1996) with slight modifications (see 2.1.1).

Protein oxidation in the absence of lipids

Whey (Lacprodan® DI-9224, Arla Foods), hazelnut and soybean protein oxidation was induced using HOCl, a strong oxidizing agent. A 200 µl protein solution (10 mg/ml) was oxidized with 50 µl HOCl (0 – 200 mM) at 30°C for 10 min. After incubation samples were immediately immersed in ice in order to stop the reaction.

Protein oxidation in the presence of lipids

The 1 % (w/w) soybean oil-in-water emulsions were prepared in 50 mM MOPS buffer pH 7.4 by homogenizing 1 % (w/w) oil with 2% (w/w) proteins at 10000 rpm for 1 min using the Ultra Turrax. The emulsions in sealed falcon tubes were oxidized at 50°C in the presence of 10 µM copper sulphate solution and 0.2 g/l sodium azide as antimicrobial agent. Defatting was performed with 3 times the volume of the model system with technical hexane. The defatted fraction was finally diluted to a known volume with PBS and protein content was determined.

Maillard reaction in the absence of other proteins

Maillard reaction was studied in a model system consisting of 10 mg/ml protein and 60 mg/ml glucose in phosphate buffer 0.1 M which was heated at 70°C for up to 48 hours. After incubation, samples were cooled immediately on ice.

Maillard reaction in the presence of other proteins

Maillard reaction was also studied in the presence of soluble wheat proteins. One model system consisted of 2.5 mg/ml whey, hazelnut or soybean proteins and 7.5 mg/ml soluble wheat proteins incubated with 60 mg/ml glucose. Another model system consisted of 5 mg/ml whey, hazelnut or soybean proteins and 5 mg/ml soluble wheat proteins. The model systems were incubated in phosphate buffer 0.1 M, pH 7.4 for 48 h at 70°C. After incubation samples were cooled immediately on ice to stop the reaction.

Protein hydrolysis

Proteins were subjected to a peptic hydrolysis with 1:250 pepsin/protein (w/w). The hydrolysis was stopped at different sampling points by increasing the pH to 6.

- ***Assessment of the chemical modifications***

Protein carbonyls

Protein carbonyls were assessed using the 2,4-dinitrophenyl-hydrazine (DNPH) assay. The conventional spectrophotometric method was used for which 300 µl oxidized proteins were incubated for 1 h with 400 µl DNPH 10 mM in 2 M HCl. The proteins were precipitated with 10 % trichloroacetic acid (TCA, final concentration) and the pellet was washed with 1 ml ethanol/ethyl acetate (1:1) to remove unreacted DNPH. The final pellet was redissolved in 500 µl 6 M urea for hazelnut and whey proteins and in 500 µl 8 M urea for soybean proteins. Finally the absorbance was measured at 370 nm. The carbonyl content was calculated using a molar absorption coefficient of 22000 M^{-1} cm^{-1} and results were expressed as nmol carbonyls /mg net protein.

Free and total thiol groups

The oxidation of the sulphur containing amino acids was monitored using 5,5'-dithiobis-(2-nitrobenzoate) (DTNB) as previously described by Beveridge et al. (Beveridge et al., 1974). Total thiol groups were determined in a similar manner after a previous reduction of the disulfide bridges with 2-mercaptoethanol. Free and total thiol groups were calculated using molar absorption coefficient of 13600 M^{-1} cm^{-1} and results are expressed as µmol SH groups/g net protein.

SDS-PAGE

Protein cross-linking or fragmentation was analyzed by SDS-PAGE under reducing conditions. A stacking gel with 4 % acrylamide and a resolving gel with 10 % acrylamide were used. Samples were prepared with mercaptoethanol and

heated at 95°C for 5 min then directly loaded on the gel. Krypton fluorescence staining was used for bands visualisation.

Available lysine

The amount of available lysine was monitored using derivatisation with *ortho*-phthaldialdehyde (OPA) in the presence of 2-mercaptoethanol which yields a fluorescent product with a maximum excitation wavelength of 340 nm and emission 450 nm (Ferrer et al., 2003). A standard of β-casein was used to prepare a calibration curve and samples were pre-treated and analyzed using a fluorimeter as previously described by Morales F.J. et al. (Morales et al., 1996).

Free amino groups

Free amino groups were determined using trinitrobenzenesulfonic acid (TNBS) as previously described by Fields R. (Fields, 1971) and results were expressed as nmol amino groups/mg protein using the molar absorbance coefficient of the TNB complex which is 22000 M^{-1} cm^{-1}.

Protein determination

Protein determination was performed by determining the nitrogen according to the Kjeldahl procedure. A factor of 6.38 was used to convert nitrogen to whey protein, 5.71 to convert nitrogen to soybean protein and 5.41 to convert nitrogen to hazelnut protein. The non protein nitrogen (NPN) was determined in the supernatant after a previous protein precipitation with 15% TCA (final concentration).

Assessment of the lipid oxidation degree

Soybean oil was oxidized under UV light (235 nm) at 35 °C. Oxidation degree of the oils used in the reaction systems was followed by iodimetric determination of the peroxide value according to the AOCS official method Cd 8-53. The secondary oxidation products of the oils used in the reaction systems were determined by measuring the p-anisidine values according to the AOCS official method Cd 18-90.

Fatty acids composition

The determination of fatty acids composition was done by means of gas chromatography based on the American Oil Chemist's Society Official Method Ce 1b-89. The triacylglycerols were saponified with a methanolic NaOH solution. Subsequently the fatty acids were esterified with BF_3/MeOH – reagent in the presence of sodium hydroxide. The methyl esters were further separated using gas chromatographic equipment (Agilent Technologies 6890N).

o Results

Protein oxidation in the absence of lipids

Formation of carbonyls is one of the most prominent changes in proteins due to oxidation and the spectrophotometric method used for their determination is the most common method used to evaluate protein oxidation in foodstuffs.

Many sources indicate that pH has an important influence of the protein oxidation; therefore the pH of the protein mixture during oxidation was monitored in order to make sure that it stays stable for all the HOCl concentrations used. Several studies (Hawkins et al., 2003) concluded that $^{-}$OCl is more reactive than the protonated acid (HOCl) and reacts more readily with the amine groups therefore the maximal rates of reaction occur at pH values that are the average of the pK_a values for HOCl and amines (~ 8.5).

To test the pH effect on protein oxidation experiments were performed in phosphate buffers 0.1 M of pH 5.8 and 8. Further, the formation of protein carbonyls and oxidation of free and total thiol groups were determined. Non-oxidized hazelnut protein isolate contained about 1,82±0,49 nmol of carbonyls/mg protein (Figure 11). As shown in Figure 11, the carbonyl content in hazelnut protein isolate increased with increasing HOCl concentration and constituted 39,84±2,30 and 40,68±1,04 nmol of carbonyls/mg proteins at pH 8 and pH 5,8 ,respectively, when 5 mmol HOCl/g protein were used. However, no significant difference between the carbonyls formed in pH 8 and pH 5,8 could be seen. Non-oxidized soybean protein isolate contained about 1,01±0,21 nmol of carbonyls/mg proteins in phosphate buffer pH 8 and 1,08 ±0,08 nmol of carbonyls/mg proteins in phosphate buffer pH 5,8. As shown in Figure 12, the carbonyl content of oxidized soybean protein isolates increased almost linearly with increasing HOCl concentration up to 55,89±1,94 nmol of carbonyls/mg proteins and 52,51±4,94 nmol of carbonyls/mg proteins at pH 8 and pH 5,8 respectively. Similar as for hazelnut protein isolate, no significant difference between the amounts of protein carbonyls formed for the two buffers used could be seen.

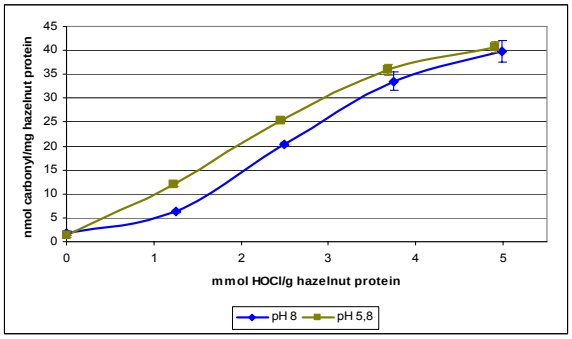


Figure 11: Effect of the pH and HOCl concentration on the formation of protein carbonyls in hazelnut isolates; the data are mean values ±SD of three independent determinations

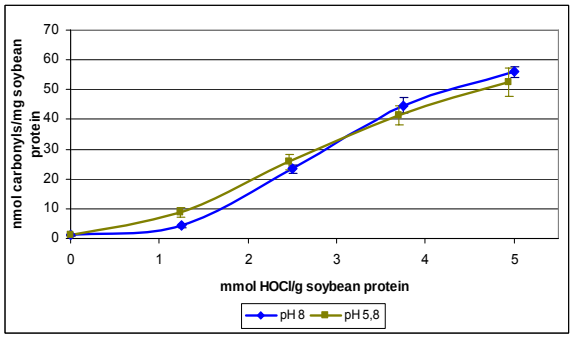


Figure 12: Effect of the pH and HOCl concentration on the formation of protein carbonyls in soybean isolates; the data are mean values ±SD of three independent determinations)

On the other hand thiols are crucial targets for oxidation. Conversion of sulfhydryl groups into disulfides and other oxidized species is one of the earliest observable events during oxidation of proteins. Ellman method is a simple, rapid and direct method to determine the SH and –S–S– group content in proteins. The Ellman spectrophotometric method is based on the reaction of DTNB with the free thiol groups in a protein and formation of a disulfide bond with releases of a thiolate ion which is coloured and has a maximal absorbance at 412 nm.

The loss of total thiol groups in hazelnut protein isolate due to HOCl induced oxidation was more prominent at pH 5,8 then at pH 8 (Figure 13). The total SH groups contents of non oxidized hazelnut protein were 104,58±1,30 µmol/g proteins at pH 8 and 85,85±0,91 µmol/g proteins at pH 5.8. Non-oxidized soybean protein in pH 8 contained 15,89±0,68 µmol free SH groups/g proteins while in pH 5,8 contained 10,93±0,09 µmol free SH groups/g proteins. Free thiol groups were completely oxidized after oxidation with 1,25 mmol HOCl/g protein. For the total thiol groups 50 % decrease was registered after oxidation in phosphate buffer pH 8 with 5 mmol HOCl/g protein and for pH 5,8 they were almost completely oxidized (Figure 13).

Total SH groups of soybean protein decrease drastically after the protein isolate was oxidized with different concentration of HOCl (Figure 14). The total SH group's content of non-oxidized soybean protein was lower at pH 5,8 (98,12±5,76 µmol/g proteins) then at pH 8 (112,95±11,24 µmol/g proteins). Free SH groups also decrease during protein oxidation (Figure 14) in both buffers. Non-oxidized soybean protein isolate in pH 5,8 contained 10,54±0,13 µmol free SH groups/g proteins while at pH 5,8 contained 4,16±0,48 µmol free SH groups/g proteins. Similarly as for hazelnut protein isolate, the decrease of free and total thiol groups is more drastic at pH 5.8 than at pH 8. This is however in contradiction with the literature data which indicate that higher pHs lead to more severe protein oxidation. The reason for the more drastic changes in protein sulfhydryl groups at pH 5.8 could probably be due to conformational changes in the protein at different pHs. Consequently, this makes the thiol groups more accessible to the HOCl attack when they are oxidized in phosphate buffer pH 5,8 compared to oxidation in phosphate buffer pH 8.

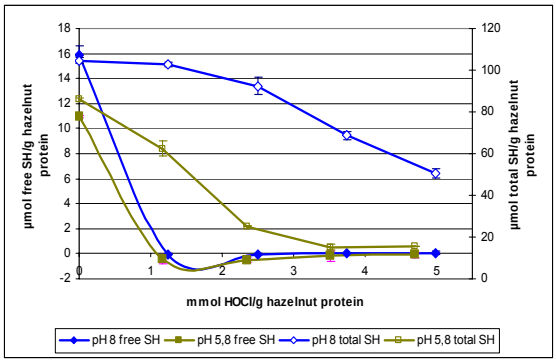


Figure 13 : Loss of free and total SH groups in hazelnut protein isolate due to oxidation with different concentration of HOCl in buffer pH 8 and pH 5,8; the data are mean values ±SD of three independent determination

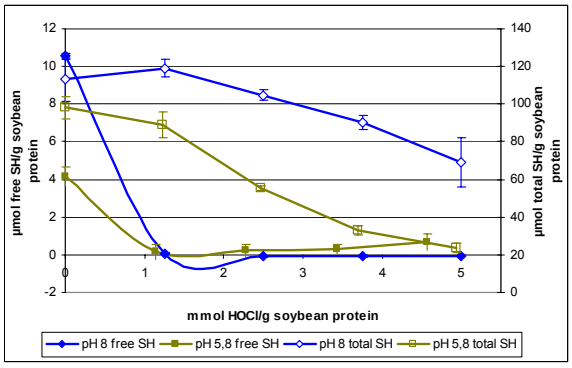


Figure 14: Loss of free and total SH groups in soybean protein isolate due to oxidation with different concentration of HOCl in buffer pH 8 and pH 5,8; the data are mean values ±SD of three independent determination

During oxidation $-NH_2$ groups of amino acids residues can be readily converted into carbonyls through a deamination process (Levine et al., 1994). On the other hand, loss of free amino groups in the oxidized proteins may also be a result of reaction of the amino groups with carbonyls which in turn leads to their decrease.
The loss of free amino groups in hazelnut protein isolate oxidized with different HOCl concentrations was more prominent at pH 8 then at pH 5,8. A decrease from 18,38±2,87 nmol of free amino groups/mg protein till 6,35±1,31 was recorded at pH 5,8 and till 2,43±1,11 nmol of free amino groups/mg protein at pH 8 (Figure 15). In the case of free amino groups loss in soybean protein isolate due to oxidation with HOCl in different pHs, in contrast to the results obtained for free and total thiols groups, the loss of free amino groups was again more prominent at pH 8 then at pH 5,8 (Figure 16). This is in good agreement with the literature data which indicate that higher pHs during HOCl induced oxidation leads to more severe changes in proteins.

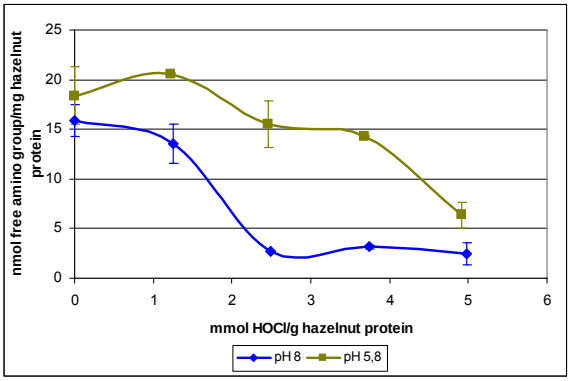


Figure 15 : Loss of free amino groups in hazelnut proteins isolates after oxidation with different HOCl concentrations in buffer pH 8 and pH 5.8; the data are mean values ±SD of three independent determinations

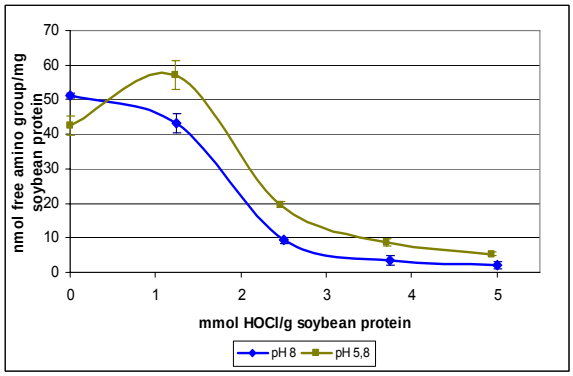


Figure 16: Loss of free amino groups in soybean proteins isolates after oxidation with different HOCl concentrations in phosphate buffers pH 8 and pH 5.8; the data are mean values ±SD of three independent determinations

The SDS-PAGE pattern of the soybean protein oxidized with HOCl indicates that the intensity of some major protein bands is decreasing (Figure 17). However no additional bands indicating smaller aggregate formation or fragmentation of the protein could be seen. Moreover, high molecular weight aggregates formed due to HOCl induced oxidation were not able to enter the gel.

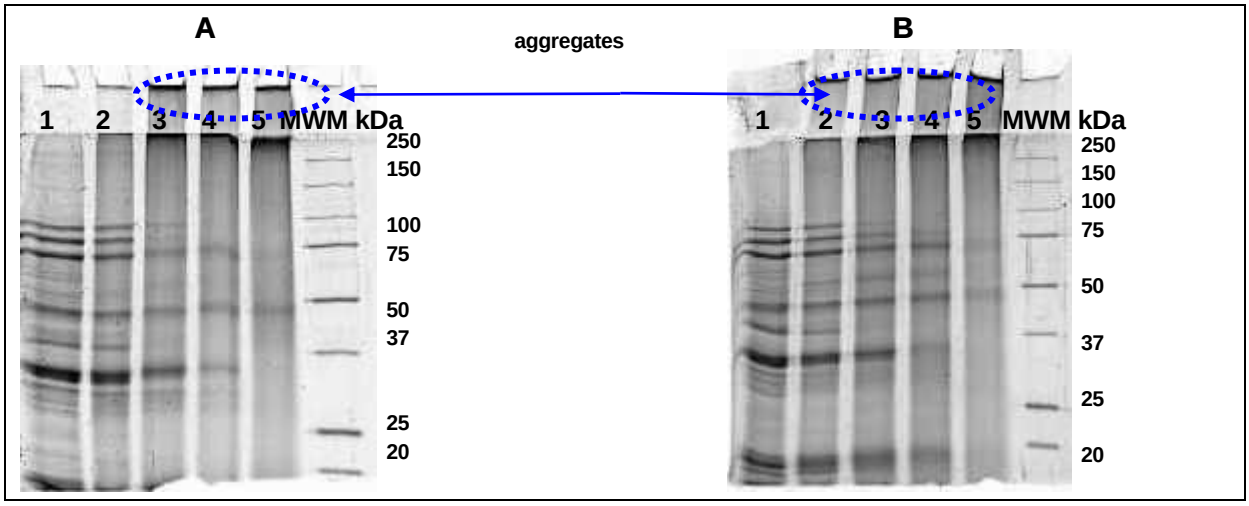


Figure 17: SDS PAGE pattern of the soybean protein oxidized with HOCl in phosphate buffer 0.1M pH 8 (A) and pH 5.8 (B). Lane 1 – soybean oxidized with 0 mmol HOCl/g protein, lane 2 - soybean oxidized with 1.25 mmol HOCl/g protein, lane 3- soybean oxidized with 2.5 mmol HOCl/g protein, lane 4 - soybean oxidized with 3,75 mmol HOCl/g protein, lane 5 - soybean oxidized with 5 mmol HOCl/g protein, lane 6 – molecular weight marker (MWM).

The SDS-PAGE pattern of the hazelnut protein oxidized with HOCl is similar to the one obtained for soybean protein with formation high molecular weight aggregates due to oxidation (Figure 18).

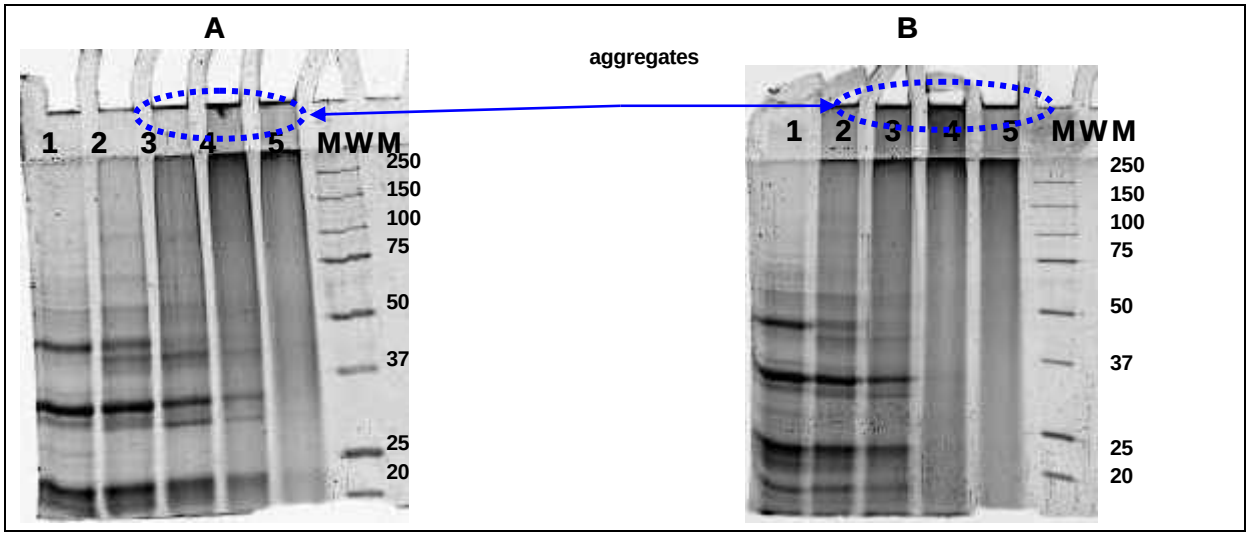


Figure 18: SDS PAGE SDS PAGE pattern of the hazelnut protein oxidized with HOCl in phosphate buffer 0.1M pH 8 (A) and pH 5.8 (B). Lane 1 – hazelnut oxidized with 0 mmol HOCl/g protein, lane 2 - hazelnut oxidized with 1.25 mmol HOCl/g protein, lane 3- hazelnut oxidized with 2.5 mmol HOCl/g protein, lane 4 - hazelnut oxidized with 3,75 mmol HOCl/g protein, lane 5 - hazelnut oxidized with 5 mmol HOCl/g protein, lane 6 – molecular weight marker (MWM)

Protein oxidation in the presence of lipids

Association of lipids with proteins occurs during the preparation of the isolate and is favoured by the denaturation of the proteins during the process. This denaturation results in exposure of the hydrophobic amino acids, hidden in the native protein, and the subsequent association between the aliphatic chains of

lipids and these amino acids by hydrophobic unions. Lipids associated with protein isolates are susceptible to oxidation during processing and storage of the products. Lipid oxidation leads to formation of many compounds such as hydroperoxides and their secondary degradation products (aldehydes, ketones, hydrocarbons, etc.). These compounds are responsible for unacceptable flavours and cause many changes in the functional and nutritional properties of the isolates as a result of their covalent interaction with proteins. Several amino acids, mainly cysteine, methionine, histidine, tryptophan, tyrosine, and lysine, are affected by the oxidation products of lipids, resulting in a reduced bioavailability (Sanchez-Vioque et al., 1999). In the same time, lipids associated with soy protein isolates for example were found to contribute to protein insolubility and protein oxidation (Liang, 1999).

In the presence of oxidizing lipids, protein oxidation is manifested by free radical chain reactions similar to those for lipid oxidation. The attack of reactive oxygen species on proteins leads to destruction of amino acid residues and the generation of carbonyl compounds. This formation is one of the most salient changes in oxidized proteins (Salminen et al., 2006). Therefore, to study the effect of fresh and oxidized soybean oil on the oxidative modifications of whey, hazelnut and soybean protein isolates, incubated with oils, the carbonyl content of the proteins was measured by derivatization with DNPH. This is the most common method used to evaluate protein oxidation in foodstuffs. Carbonyl compounds derived from lipid oxidation may increase the protein carbonyl values in the DNPH assay. Therefore, proteins were first defatted prior to the DNPH assay. Beside this, they were precipitated and thoroughly washed after the derivatization, hence any unreacted DNPH or DNPH bound to any other component than protein was removed.

Soybean oil oxidation was induced by UV light at 35°C.This allowed to evaluate the impact of oils with different oxidation degrees on protein oxidation. It is generally accepted that the first compounds formed during oxidation of oil are hydroperoxides. The usual method of hydroperoxide assessment is by determination of the Peroxide Value. The peroxides in oxidized oil are transitory intermediates, which decompose into carbonyls and other compounds. P-anisidine assay measures the level of aldehydes, principally 2-alkenals, present in the oils.

indicates the p-anserine value and peroxide value of the oils used for the reaction systems while Table 8 indicates the fatty acids composition of the oils.

Table 7: Peroxide and P-anserine value of the oils used in the reaction systems

Oil type	Peroxide value (meq O_2/kg)	P-anisidine value
Oxidized soybean oil	25.00 ± 0.23	8.66 ± 0.33
Fresh soybean oil	2.02 ± 0.15	4.40 ± 1.01
Olive oil	7.85 ± 0.15	5.18 ± 0.21
Fish oil	3.27 ± 0.04	12.37 ± 0.83
Sunflower oil	1.36 ± 0.05	11.63 ± 0.26

Table 8: Fatty acid composition of the oils used in the reaction systems

Oils	Plamitoleic acid(C16:1)	Oleic acid(C18:1)	Linoleic acid(C18:2)	Linolenic acid(C18:3)	EPA (C20:5)	DHA (C22:6)
Oxidized soybean	0.102	26.207	0.432	6.341	0.156	-
Fresh soybean	0.103	25.771	0.407	6.417	0.147	-
Olive	0.787	75.262	0.020	0.827	0.072	-
Fish	7.916	13.927	5.689	0.978	18.124	12.001
Sunflower	5.889	32.432	0.198	0.153	0.248	-

The measurements of protein oxidation demonstrate that model systems with fresh fish oil led to the formation of highest level of carbonyls after 120 h incubation with hazelnut, soybean and whey proteins (Figure 19, Figure 20 and Figure 21). Fish oils are rich in polyunsaturated fatty acids which are susceptible to oxidation, therefore, leading to the formation of their peroxides. Due to this, their presence in protein food can result in oxidation and protein damage (Saeed and Howell, 1999). It is evident from the data that mainly polyunsaturated oils (sunflower and fish oil) were able to stimulate carbonyl formation of proteins (Figure 23). As previously reported by (Refsgaard et al., 2000), the ability of fatty acids to form protein carbonyl formation is strongly dependent on the degree of unsaturation and increases with the degree of unsaturation, in the order C18:2< C18:3< C20:4. The metal-catalyzed oxidation of the polyunsaturated fatty acids is leading to the formation of several products that have been shown to form carbonyls derivatives with proteins. These include malondialdehyde, α, β – unsaturated aldehydes and lipid peroxides which can be converted to alkoxyl and peroxyl radical that can react directly with side chains of some amino acids residues to form carbonyl derivatives. Olive oil, which contains mainly monounsaturated fatty acids, led to the formation of very little amounts of protein carbonyls even after 120 h incubation at 50°C.

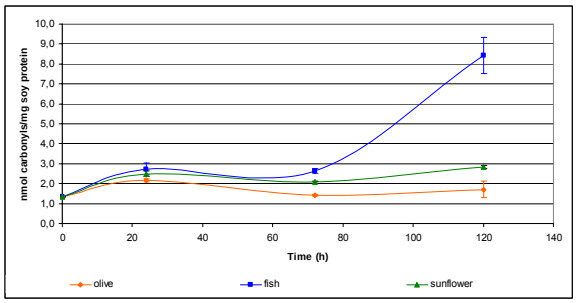


Figure 19 : Formation of carbonyls in soybean protein isolates incubated with oils with different degrees of unsaturation, the data are mean values ±SD of three independent determinations

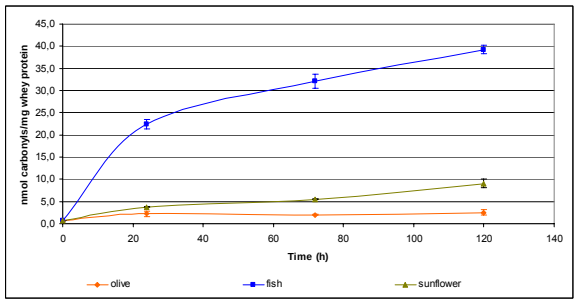


Figure 20: Formation of carbonyls in whey protein isolates incubated with oils with different degrees of unsaturation, the data are mean values ±SD of three independent determinations

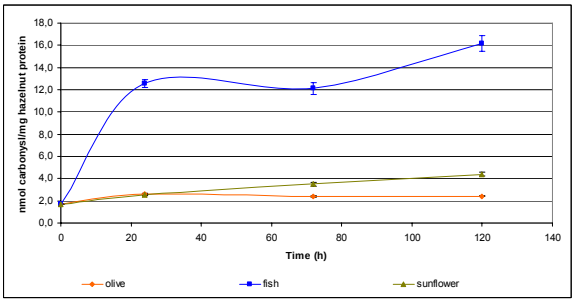


Figure 21: Formation of carbonyls in soybean protein isolates incubated with oils with different degrees of unsaturation, the data are mean values ±SD of three independent determinations

The reaction between the breakdown products of peroxides and proteins has received considerable attention over the last years. It is well known that end-products of lipid peroxidation, such as malondialdehyde and 4 – hydroxy – nonenal, cause protein damage by means of reactions with amino groups, sulfhydryl groups and imidazole groups of amino acids. To test the possibility that lipid hydroperoxides formed during the oxidation of oils contribute to the generation of protein carbonyls, we incubated protein isolates with oils with different oxidation degrees (see Table 7). Incubation of whey and soybean protein isolate with oxidized soybean oil led to a substantial increase in the level of protein carbonyls (©Figure 22,Figure 23). However in the case of hazelnut protein isolate incubated with oxidized oil no significant increase in the amount of protein carbonyls could be seen as compared to the reaction system incubated with fresh oil (Figure 24). This is probably due to the fact that the amount of hydroperoxides and secondary oxidation products is not high enough to induce formation of such high level of protein carbonyls.

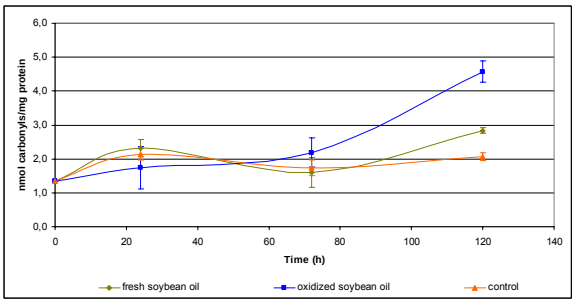


Figure 22: Formation of carbonyls in soybean protein isolates incubated with oils with different oxidation degrees, the data are mean values ±SD of three independent determinations

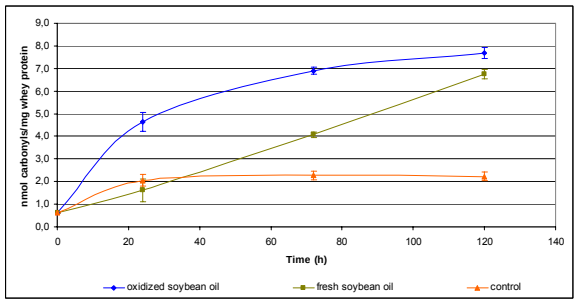


Figure 23: Formation of carbonyls in whey protein isolates incubated with oils with different oxidation degrees, the data are mean values ±SD of three independent determinations

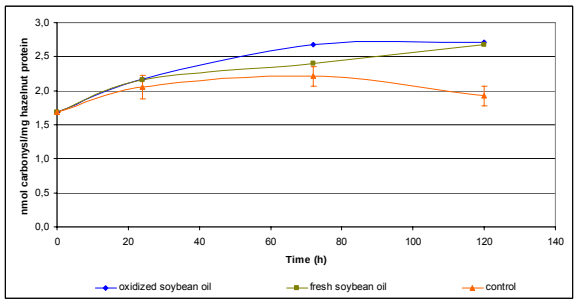


Figure 24: Formation of carbonyls in whey protein isolates incubated with oils with different oxidation degrees, the data are mean values ±SD of three independent determinations

The determination of free and total thiol groups of the protein isolates during incubation with lipids is of outmost importance since thiols are crucial targets for oxidation. As a result of oxidation protein-protein interactions may occur and lead to intra- or intermolecular thiol/disulfide interchange or thiol/thiol oxidation reaction (Monahan et al., 1996). After 72h incubation of hazelnut, soybean or whey protein isolates with oils, all free thiol groups were oxidized. The oxidation of free thiol groups could imply that proteins were denatured after reaction, aggregated through intermolecular disulfide bonds, or oxidized. However, a decrease in the total thiol groups was also observed (Figure 15, 16 and 17). Huang et al. (2006) also reported degradation of total thiol and free thiol groups in soybean during oxidation with linoleic acid and suggested that the process of sulfhydryl-disulfide changes did not occur in the reaction because some cysteine might be lost due to oxidation of sulfhydryl to other groups then disulfide bonds (Huang et al., 2006). The decrease of free and total thiol groups was not highly dependent on the unsaturation degree of the oils used in the case of hazelnut and soybean protein isolates. However the initial decrease was more rapid in the case of fish oil than in the case of other oils used. In the case of whey protein the decrease of total thiol groups was more prominent when fish oil was used in the reaction system. The decrease of total thiol groups was not either dependent on the oxidation degree of

the oils used (data not shown). One of the reasons might be the fact that the oxidized soybean oil contained a rather low amount of secondary oxidation products.

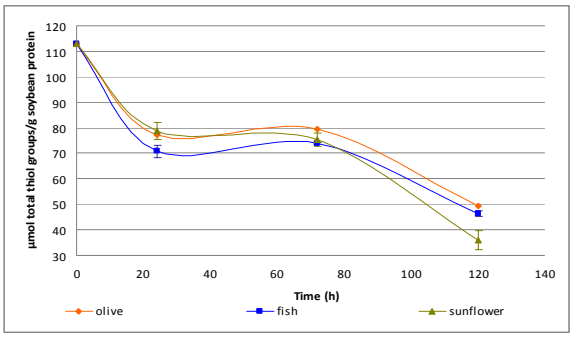


Figure 25: Loss of total thiol groups in soybean protein isolates incubated with oils with different unsaturation degrees, the data are mean values ±SD of three independent determinations

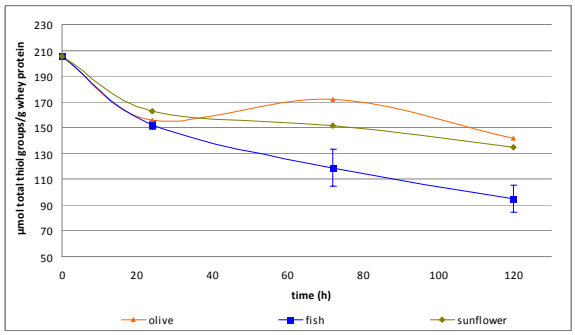


Figure 26: Loss of total thiol groups in whey protein isolates incubated with oils with different unsaturation degrees, the data are mean values ±SD of three independent determinations

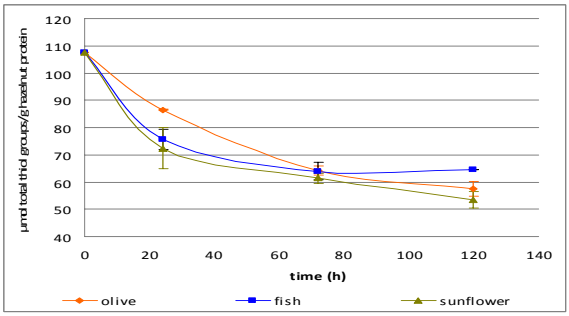


Figure 27: Loss of total thiol groups in hazelnut protein isolates incubated with oils with different unsaturation degrees, the data are mean values ±SD of three independent determination

According to Refsgaard et al. (2000), the lysine residues are among the preferred for reaction with all types of lipid peroxidation products (Refsgaard et al., 2000). Moreover, he suggested that the loss of lysine residues increases as a function of the degree of fatty acid unsaturation. In addition to reactions that yield carbonyl derivatives, lysine residues of proteins might react with lipid derived aldehydes to form Schiff base derivatives. Our results indicate that the loss of lysine is the most prominent in the case of protein isolates incubation with fish oil. No significant difference between the losses of available lysine could be seen when the protein isolates were incubated with sunflower and olive oil (Figure 28, Figure 29 and Figure 30). In the same time no significant difference between the losses of available lysine losses during incubation with fresh or oxidized soybean oil could be seen.

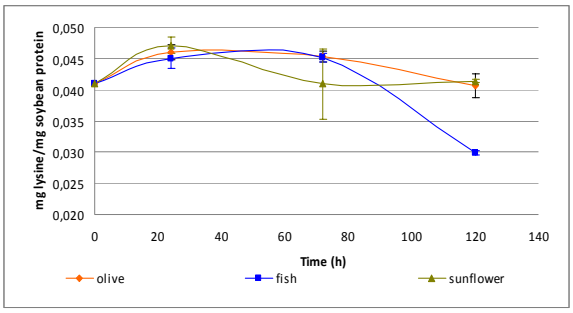


Figure 28: Loss of available lysine in soybean protein isolates incubated with oils with different unsaturation degrees, the data are mean values ±SD of four independent determinations

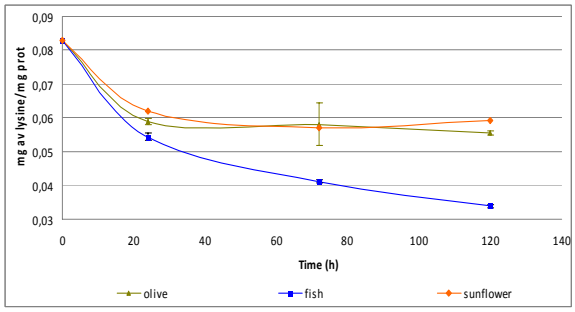


Figure 29: Loss of available lysine in whey protein isolates incubated with oils with different unsaturation degrees, the data are mean values ±SD of four independent determinations

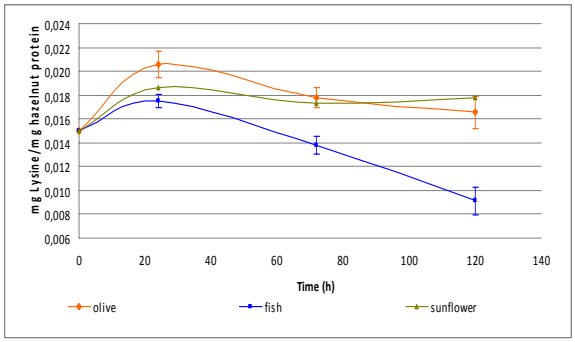


Figure 30: Loss of available lysine in hazelnut protein isolates incubated with oils with different unsaturation degrees, the data are mean values ±SD of three independent determinations

Besides all the protein damages indicated above, incubation of protein isolates with oils, led to proteins precipitation. This decrease in solubility is a common manifestation of damage to proteins exposed to oxidizing lipids which is in good agreement with the literature data. This loss of solubility could have been caused by the formation of lipid protein complexes, by protein aggregation initiated by free radical reactions with the lipids or by reactions with the reactive decomposition products of lipids.

Maillard reaction in the absence of other proteins

Literature data indicate that Maillard type reactions taking place during processing of foods might influence IgE-binding properties of food allergens (Gruber P. et al., 2004). To investigate the influence of the Maillard reaction on the allergens detectability by the available commercial methods, they were thermally treated in the presence or absence of glucose (10mg protein/ml with 60 mg glucose/ml).

Maillard reaction was induced by thermally treating protein isolates (10 mg/ml) with 60 mg glucose/ml in phosphate buffer 0.1M pH 7.4. It is known that reactive dicarbonyl compounds originating from Maillard reaction can lead to the formation of protein bound carbonyls. Therefore an increased level of carbonyls besides being a useful marker for the protein oxidation, it can also be useful marker of the extent of the Maillard reaction. Carbonyl compounds derived from carbohydrates may increase the protein carbonyl values in the DNPH assay. Therefore proteins were precipitated and thoroughly washed after derivatization; hence any unreacted DNPH or DNPH bound to any other components than protein was removed. Thermal treatment of whey, hazelnut and soybean protein isolates with glucose led to protein carbonyl formation which increased with increasing incubation time (figure 21). The amount of carbonyls increased linearly when whey protein was thermally treated with 60 mg/ml glucose. In the case of whey and soybean protein isolates the amount of protein carbonyls increased linearly during 42 h after which a slight decrease in protein carbonyls was seen. This is probably due to polymerisation that takes place during longer incubation times. When no glucose was used during incubation, the amount of carbonyls did not increase.

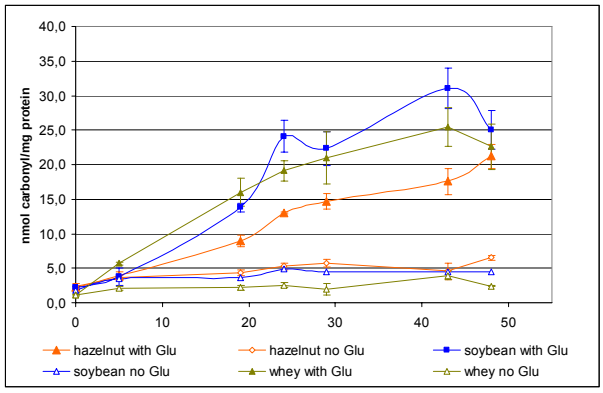


Figure 31: Protein carbonyls formation in hazelnut, soybean and whey protein isolates incubated with or without glucose, the data are mean values ±SD of three independent determinations

A continuous decrease in free thiol groups was also registered when whey protein was thermally treated with and without glucose. However no significant difference between the decreases in samples incubated with or without glucose could be seen. On the other hand the decrease of total thiol groups was more prominent when reaction systems were incubated in the presence of glucose (figure 22). After incubation of protein isolates with glucose a 62 % decrease in total thiol groups was observed in the case of soybean protein isolate. In the case of whey and hazelnut proteins the decrease was less drastic with 52 and 31 % decrease respectively.

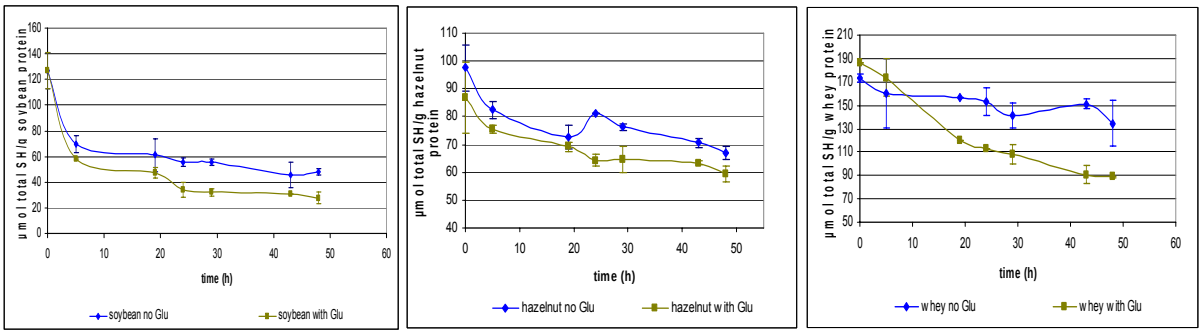


Figure 32: Loss of total thiol groups in hazelnut, soybean and whey protein isolates incubated with or without glucose, the data are mean values ±SD of three independent determinations

Loss of free amino groups is one of the main indicators of the extent of the Maillard reaction. A time dependent decrease in free amino groups was therefore observed in the case of hazelnut, soybean and whey protein isolates incubated with glucose (figure 23 and 24). The initial amount of available lysine in hazelnut protein isolate is much lower than in the soybean and whey protein isolate.

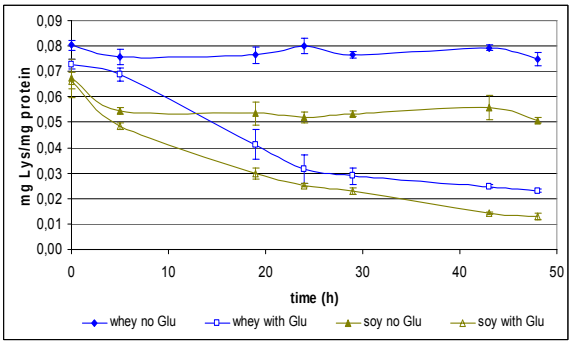


Figure 33: Loss of available lysine in soybean and whey protein isolates incubated with or without glucose, the data are mean values ±SD of three independent determinations

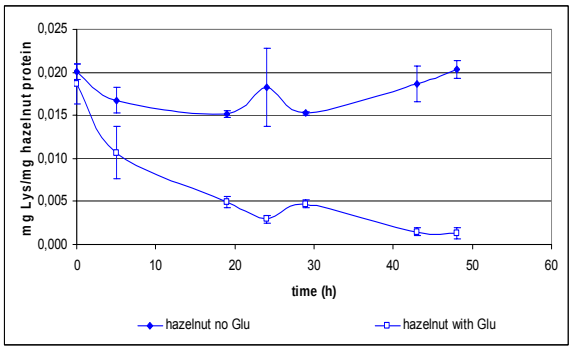


Figure 34: Loss of available lysine in hazelnut protein isolates incubated with or without glucose, the data are mean values ±SD of three independent determinations

The SDS-PAGE pattern of the hazelnut, soybean and whey protein isolates thermally treated with and without 60 mg/ml glucose shows aggregates formation represented by band smearing (figure 24). The aggregates are already formed after 5 h incubation of the whey and soybean protein isolate. In the case of hazelnut protein isolate incubated with glucose the aggregates are formed much later (after 24- 29 h incubation).

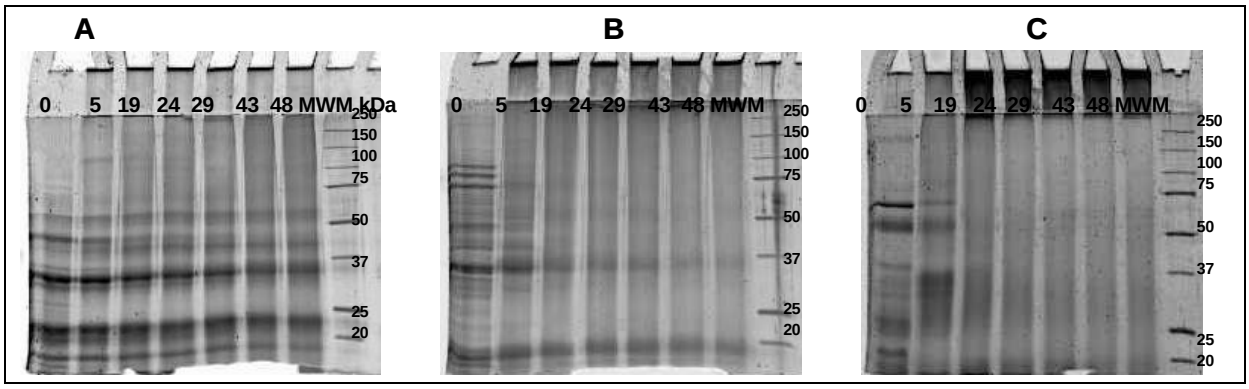


Figure 35: SDS PAGE pattern of the hazelnut (A) soybean (B) and whey (C) protein isolates incubated with glucose for: lane 1 –0 h, lane 2 -5 h, lane 3 – 19 h, lane 4 – 24 h, lane 5 -29 h, lane 6 – 43 h, lane 7 – 48 h, lane 8 - molecular weight marker (MWM).

Maillard reaction in the presence of soluble wheat proteins

During the Maillard reaction, proteins are subjected to dramatic changes in their structure. Apart from changes on amino acid level (such as available lysine losses) also cross linking may occur between different proteins. Therefore, the reaction is also studied in presence of other food proteins. It is clear that the other proteins are the bulk proteins present in the food (such as wheat flour). It is possible that when allergens are present in the food due to cross contamination, their concentrations will be low compared to the concentration of other proteins. By cross-linking with other proteins, the allergen epitopes may become buried and thus not accessible for the antibodies used in immunochemical detection methods. Therefore the influence of Maillard reaction in the presence of other proteins on the immunochemical detection by the commercial available methods will also be assessed. Two model systems consisting of mixture of soluble wheat proteins and the respective protein isolate were used. One model system contained 5 mg/ml and another 7.5 mg/ml wheat protein respectively. The amount of protein carbonyls and losses of available lysine were assessed (Figure 36,Figure 37). Besides this, losses of total thiol groups after incubation with glucose were registered. The results obtained represent the changes on the entire protein mixture. The main goal is however to assess the impact of the presence of other proteins in the reaction system on the immunochemical detection.

As can be seen from Figure 37, the wheat protein is pour in available lysine, therefore the amounts detected are much lower than when pure protein isolates were used in reaction systems (respectively 75% and 50% less available lysine present).

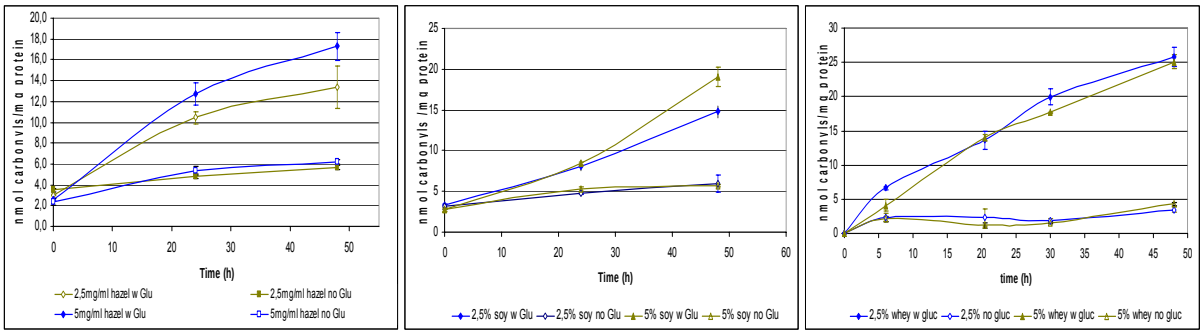


Figure 36: Protein carbonyls formation in model systems containing wheat protein with hazelnut, soybean and whey protein respectively, incubated with or without glucose, the data are mean values ±SD of three independent determinations

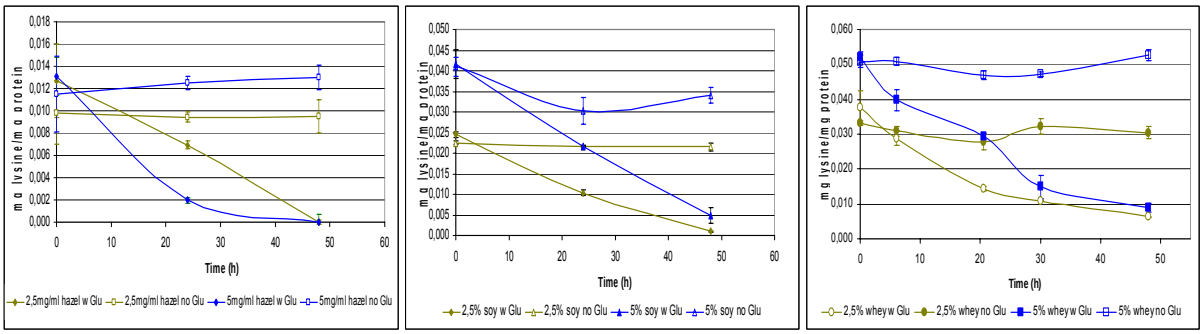


Figure 37: Loss of available lysine in model systems containing wheat protein with hazelnut, soybean and whey protein respectively, incubated with or without glucose, the data are mean values ±SD of three independent determinations

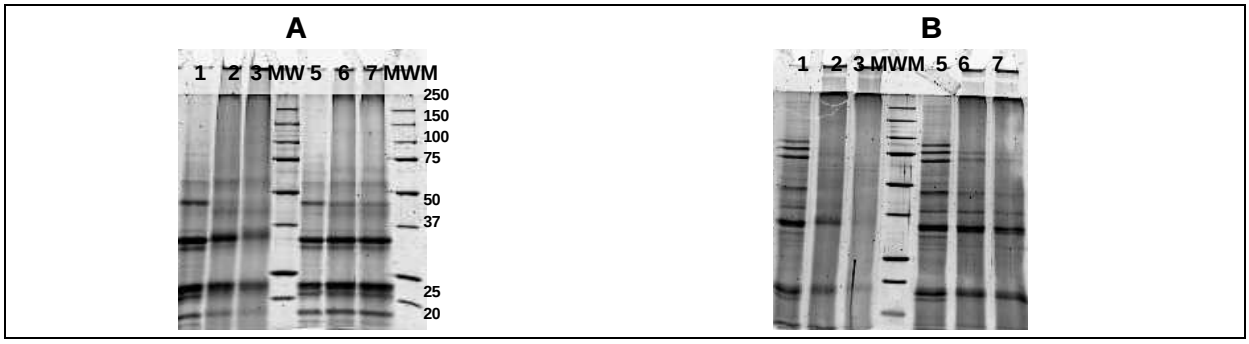


Figure 38: SDS PAGE pattern of the hazelnut (A) soybean (B) protein isolates incubated with or without glucose in the presence of soluble wheat proteins. A**: lane 1 – 0h of 5 mg / ml hazelnut with 5 mg / ml wheat proteins with glucose, lane 2 -24h of 5 mg / ml hazelnut with 5 mg / ml wheat proteins with glucose, lane 3 – 48h of 5 mg / ml hazelnut with 5 mg / ml wheat proteins with glucose, lane 4 – molecular weight marker, lane 5 -0h of 5 mg / ml hazelnut with 5 mg / ml wheat proteins without glucose, lane 6 – 24h of 5 mg / ml hazelnut with 5 mg / ml wheat proteins without glucose, lane 7 – 48h of 5 mg / ml hazelnut with 5 mg / ml wheat proteins without glucose, lane 8 – molecular weight marker.** B**: lane 1 –0h of 2.5 mg / ml soybean with 7.5 mg / ml wheat proteins with glucose, lane 2 -24h of 2.5 mg / ml soybean with 7.5 mg / ml wheat proteins with glucose, lane 3 – 48h of 2.5 mg / ml soybean with 7.5 mg / ml wheat proteins with glucose, lane 4 – molecular weight marker, lane 5 -0h of 2.5 mg / ml hazelnut with 7.5 mg / ml wheat proteins without glucose, lane 6 – 24h of 2.5 mg / ml hazelnut with 7.5 mg / ml wheat proteins without glucose, lane 7 – 48h of 2.5 mg / ml hazelnut with 7.5 mg / ml wheat proteins without glucose.**

Protein hydrolysis

Partial protein hydrolysis was induced by incubating protein isolates with pepsin in a protein-to-pepsin ratio of 1: 250. Though this will not exactly simulate a food processing step, recently protein hydrolysis is more often used to improve protein quality, remove bitterness, improve amino acid availability and modify some functional properties. Hydrolysates are recently used in products for special nutrition like diets for elderly and patients with impaired gastrointestinal absorption, hypoallergenic infant formulas, sports nutrition, and weight-control diets, as well as in consumer products for general use (de la Barca et al., 2000).

As can be seen from Figure 39 protein hydrolysis took place faster for the hazelnut protein compared to whey and soybean protein. This can be also seen from the results obtained in SDS-PAGE pattern (Figure 40), where major protein bands of the hazelnut proteins disappeared already after 15 min hydrolysis while some from the soybean protein remain stable even after 180 min incubation with pepsin. This is in good agreement with the literature data which indicate that hazelnut protein was more susceptible to hydrolysis, while soybean proteins, that contain the conglycinins, are more stable.

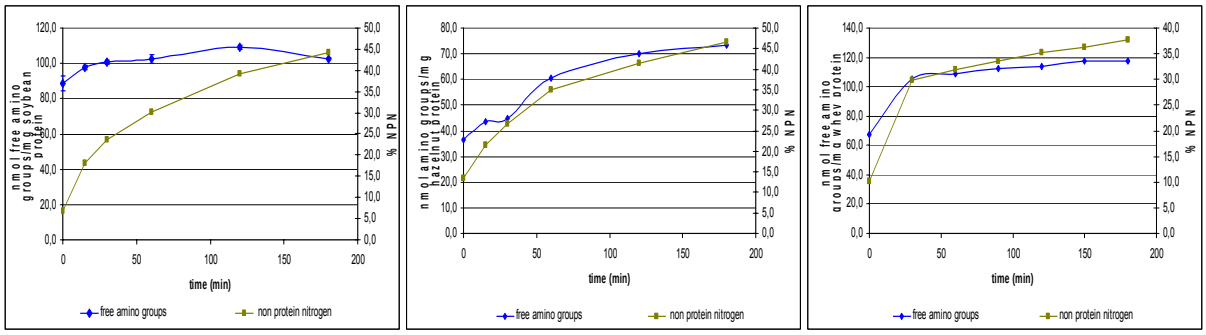


Figure 39: Extent of hazelnut protein hydrolysis as determined by the free amino group's amount and % non protein nitrogen

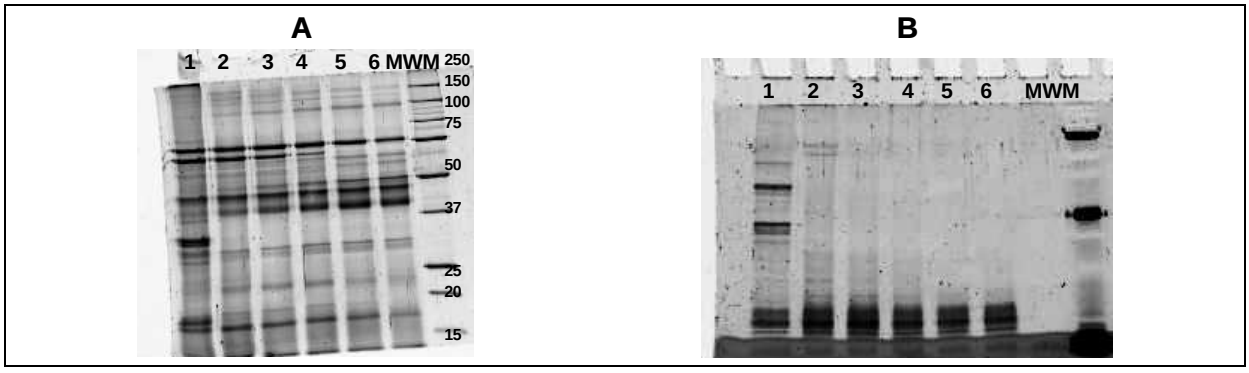


Figure 40: SDS-PAGE pattern of pepsin hydrolysed soybean (A) and hazelnut (B) protein isolates. Lane 1-non hydrolysed protein, lane 2- 15 min hydrolysis, lane 3 - 30min hydrolysis, lane 4 - 60 min hydrolysis, lane 5 – 120 min hydrolysis, lane 6 – 180 min hydrolysis, lane 7- molecular weight marker.

3.1.5. Mass spectrometric study of changes in allergen proteins by application of controlled chemical reaction

- Partner

  UGent

- Objective

In order to be able to assess the exact nature of chemical modifications of proteins as a results of their subjection to food processing simulating reactions, mass spectrometric techniques were applied.

- Methodology

After SDS-PAGE separation of the chemically modified proteins, the specific bands were cut and digested with trypsin. Peptides were extracted from gels and concentrated by vacuum centrifugation. The obtained peptides were analysed by MALDI-TOF. MALDI-TOF MS measurements were carried out using α-cyano-4-hydroxycinnamic acid as matrix. Peptide masses determined in the reflector mode were internally calibrated. Later, chemically modified proteins were also digested with trypsin without previous separation on the SDS-PAGE. Protein identification was performed by searching in the protein sequence database (Swiss-Prot/TremBL) using the Mascot search engine (http://www.matrixscience. com). On the other hand analysis by MS/MS was also performed.

- Results

Samples oxidized with HOCl in pH 5.8 and 8 were first separated by SDS-PAGE (see Figure 17). However, because high molecular weight aggregates formation as a result of oxidation were formed, most of them remained in the stacking gel without being able to pass into the separating gel. This made it difficult to cut out protein bands and perform an in-gel digestion. To overcome this issue, the chemically modified protein isolates were directly digested with trypsin (1:240 trypsin to protein ratio). Nevertheless, the number of protein peptides obtained after digestion of the chemically modified protein extracts *versus* the not-treated was too limited. This was probably due to the fact that a too high trypsin-to-protein ratio was used for the digestion, hindering complete protein digestion. Therefore, a higher amount of trypsin was used in the further experiments (namely 1:40 trypsin-to-protein ratio). The resulting number of peptides after digestion was much higher. This allowed identification of peptides of all the main allergens from soybean and hazelnut. MS/MS along with the search in the Swiss-Prot/TremBL data base allowed confirmation of the identity of the obtained peptides. Further, the protein isolates chemically modified through Maillard reaction and through lipid-induced oxidation will be analysed.

Work package 2: Evaluation of optimised immunochemical and PCR integrated analytical techniques

3.2.1. Development of a new real-time PCR

- Partner

  ILVO

- Objective

The aim of this task is to develop a new real-time PCR assay targeting one or more genes coding for allergenic proteins. As DNA is more stable, this method could be more suitable to detect hazelnut/soy after food processing.

- Methodology

Primers were developed amplifying a sequence of the genes coding for *Cor a 1*, *Cor a 8* and *Cor a 11* from hazelnut. The available nucleotide sequences of these proteins were assembled from public databases (*Cor a 1*: Z72440, *Cor a 8*: AF329829, *Cor a 11*: AF441864). Each sequence was blasted for sequence homology with other nucleotide sequences and aligned to the other allergen sequences (ClustalW software). Primers were designed on the conserved regions using the Primer 3 tool.

The specificity of the primer pairs for the specified allergen gene within the hazelnut genome was tested by a real-time PCR with SYBR-Green I and determination of the melting curve of the amplicons. To check if the primer pairs can align with a nucleotide sequence from another species, a Primer-BLAST was performed against a nucleotide database containing all GenBank + RefSeq Nucleotides + EMBL + DDBJ + PDB sequences. Specifity of the primer pairs will also be tested by PCR on DNA extracted from the sources selected to test the specificity of the ELISA kits (see Table 4).

The LOD of each developed real-time PCR assay was determined by a serial dilution of hazelnut DNA extract.

- Results

The melting curve of the amplicons derived after real-time PCR with Sybr-Green I fluorescent detection with the designed primers on hazelnut DNA extract resulted in single peaks (Figure 41, Figure 42). This means that no aspecific fragments are amplified and indicates that the primers are specific for the allergen gene sequence.

Table 9: Overview of the developed primers for genes of hazelnut allergens

Allergen	Primers	Sequence (5'-3')	Amplicon size
Cor a 1	Cor a1 F1 (forward) Cor a1 R1 (reverse) Cor a1 F2 (forward) Cor a1 R2 (reverse)	AAG ATA GTG GCA TCC CCT CA CCA GCC TTA ATC TGC TCG TC GGC AGC CCA TTC AAC TAC AT CAT GAG GGG ATG CCA CTA TC	101 bp 148 bp
Cor a 8	Cor a8 F1 (forward) Cor a8 R1 (reverse)	TGC GTG CTC TAC CTG AAG AA GTG GAG GGG CTG ATC TTG TA	218 bp
Cor a 11	Cor a11 F1 (forward) Cor a11 R1 (reverse)	GGT CCG ATC AAT CTT CTC CA GGG ACA AGC CAT TTC AAA GA	222 bp

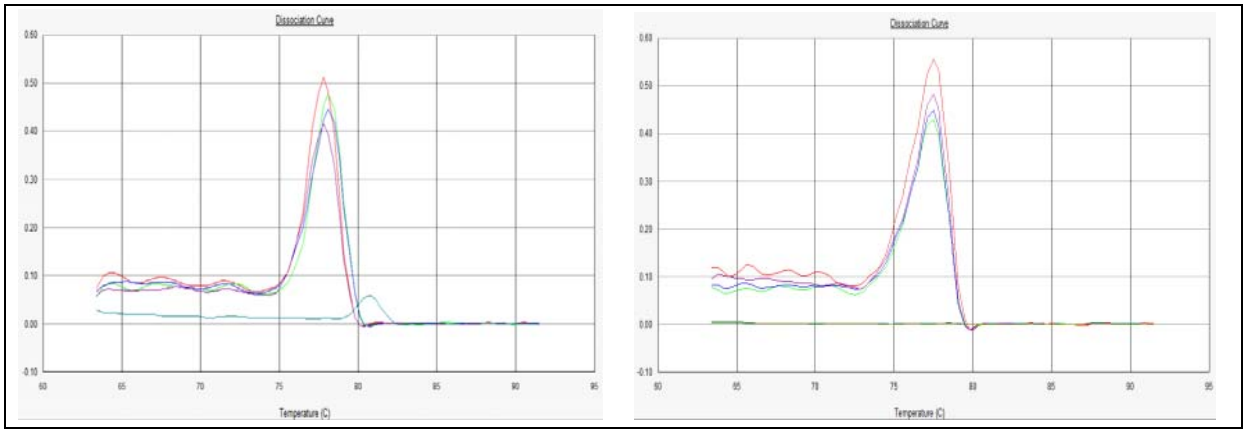


Figure 41: Real-time PCR melting piques of amplification products for Cor a1 F1/R1 (left) and Cor a1 F2/R2 primers

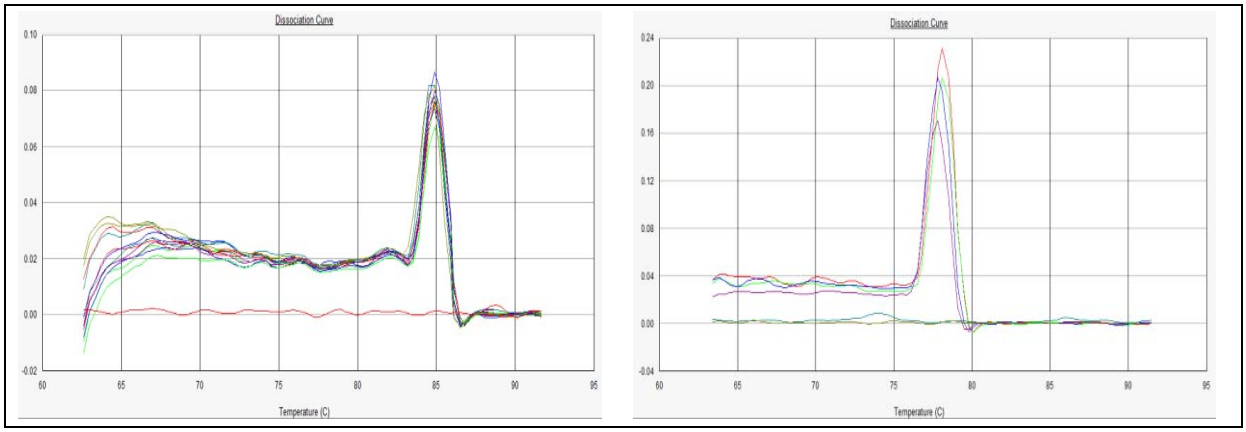


Figure 42: Real-time PCR melting piques of amplification products for Cor a8 F1/R1 (left) and Cor a11 F1/R1

Primer-BLAST with the Cor a8F1/R1 and Cor a11F1/R1 primer pairs showed 100% specificity for the template. The primer pairs designed for the *Cor a 1* gene align with the DNA sequences of four isoforms of *Cor a 1*, each time with 3 mismatches. Examination for the origin of the DNA sequence of *Cor a 1* used as

template (acc. n°. Z72440) revealed that it is derived after total genomic DNA extraction from leaves of the hazel plant (Hoffmann-Sommergruber et al., 1997). The sequences of the four isoforms, *Cor a 1.0401*, *Cor a 1.0402*, *Cor a 1.0403* and *Cor a 1.0404*, originated from RNA extraction and cDNA synthesis from immature ground hazelnuts (Luttkopf et al., 2002). As this study concerns food allergens, assays have to be developed which detect hazelnut allergens from the nuts and not from pollen or leaves of the plant. Therefore, new primer pairs were designed with the sequence of *Cor a 1.0401* (AF136945) as template. These still have to be tested in a PCR with hazelnut DNA.

Preliminary experiments were performed to test the sensitivity of the PCR assays by real-time PCR with a dilution series of DNA extract from hazelnuts. The lowest concentration that still gives an amplification plot was taken as the LOD. Further experiments with spiked matrix need to be performed in order to determine the LOD.

- Conclusion

The designed primer pairs are specific for the template within the hazelnut genome. Those for the genes of Cor a 8 and Cor a 11 are also specific for the *Corylus avellana* species, as tested with Primer-BLAST *in silico*. The template used to design the primer pairs for the DNA sequence of *Cor a 1* was obtained from DNA extraction from leaves of the hazel plant. New primers were designed using the DNA sequence of *Cor a 1.0401*, the major allergen in hazelnuts.

Work package 3: Development of quantitative mass spectrometric methods

3.3.1. Development of the heavy peptides method

- Partner

  ULiege

- Objective

The « Heavy peptides method » is a strategy for absolute quantification of proteins and post-translational modifications (Gerber et al., 2003; Kirkpatrick et al., 2005). It is a variation of isotope dilution mass spectrometry techniques commonly used for the measurement of small molecules (Bowers et al., 1993), but the difference is that instead of directly measuring the small molecule the quantification of the protein is made at the peptide level. In the food allergy field, the Heavy peptides method can be useful to provide an absolute quantification of allergens and allows the confirmation of the presence of hazelnut or soy in the food. By targeting a peptide rather than the whole protein (such as in most immunochemical methods), the efficiency of the detection is greatly increased since it is independent of the 3D-structure of the allergen. A stable isotope-labelled internal standard peptide (IS) is added to the food sample during protein extraction. Both the IS and the native unlabelled peptide (identical to the IS except its mass) are measured by LC-MS/MS. Since the amount of IS is known, and the ratio between the native peptide and the IS can be calculated from the chromatogram, the amount of native peptide in the sample can be deduced. The quantification is performed with a high confidence level due to three reasons: the detection and the recognition of peptides of interest are firstly based on a separation according to their retention time during HPLC, secondly on their own m/z ratio in MS and thirdly, on the m/z ratio of two specific transitions per peptide monitored in MS/MS.

- Results

The first part of the development of the Heavy peptide method consists of the selection of tryptic peptides of interest matching to criteria (Table 10). A first peptide is selected to be an internal standard (IS) in order to calibrate the method and eliminate the experimental variability. A second peptide is selected to be a "target peptide", representative of the allergenic material we want to quantify. We mean for allergenic material, the total allergen content of the seed (hazelnut, soy) or a given allergen of the seed. One target peptide must be selected for each material to quantify.

Table 10: Criteria for the selection of the peptides-of-interest in the Heavy peptides method, the internal standard and the target peptide.

		Tryptic peptides of interest	
		Internal standard (IS)	**Target peptide**
Criteria	1	Do not contain cysteine (avoid the formation of disulfide bonds preventing the trypsin to cleave protein)	
	2	No miscleavage	
	3	Sequence common to all variants of the protein if it is the case	
	4	Specific of anyone organism but with similar physicochemical properties than the material to quantify (more suitable if belong to the organism of interest, hazelnut or soy)	- Specific of hazelnut/soy to quantify the total amount of the seed in food. - Specific of an hazelnut/soy allergen to quantify this allergen in particular
	5	Easily detectable in MS and MS/MS (no "matrix effect" or background noise hiding the ion signal)	
	6	Specific fragments (daughter ions) produced in MS/MS	
	7	Not too long (expensive cost of synthesis)	
	8	Labelled with ^{13}C and ^{15}N isotopes (a mass difference increases the resolution in mass spectrometry and avoid the confusion with the signal of the corresponding native peptide if the latter is present in food)	

- ***Selection of the target peptide***

In practice, the lyophilised protein extracts of hazelnut and soy given by ILVO were prepared to be used in liquid chromatography and mass spectrometry. Several filter devices have been attempted to remove buffer salts, protease inhibitors and others interfering molecules in extracts. The lyophilisates were resuspended in carbonate buffer (NH_4HCO_3 100 mM) and the protein concentrations, around 3 mg/mL were measured by colorimetric *RC DC protein assay*. For hazelnut, the three filter devices compared are the membrane Amicon® 5 kDa, the membrane Microcon® 3 kDa and the size exclusion chromatography column Micro Bio-Spin™ 6 kDa. For Soy, a first attempt has been done with the membrane Biomax® 5 kDa.

The choice of the filter device is based on the diversity of peptides identified in LC-MS/MS for a given protein. For the hazelnut protein *Cor a 9* for instance, more peptides and parent ions are identified with the Amicon® device. For soy, results are in progress. A 2-D Clean-Up kit was also employed after the first filtration in order to remove remaining salts and non-protein contaminants such as lipids, phenolics or nucleic acids. It has been noticed that the clean-up improves the quality of results, so this step is maintained. The in-solution digestion was

performed according to the protocol of Kim et *al.*, 2006 (Kim et al., 2006): solubilization of proteins with ammonium bicarbonate buffer NH_4HCO_3 100 mM pH8, sonication 3 x 20 seconds to break potential protein aggregates, reduction (30 min at 56°C with DTT 20mM) and alkylation (30 min at RT with IAA 60mM) in order to ensure an efficient denaturation of proteins, digestion for 16h with recombinant trypsin, second reduction and alkylation steps, second digestion phase for 4h with new trypsin. Three different modes of denaturation during the reduction step of proteins have been investigated using the sample desalted with the Amicon® device: with DTT at 56°C, with DTT at RT, and without DTT/with urea at RT. The best sample preparation is to denature proteins with DTT at RT, more peptides and parents ions are identified because they are not destroyed by heat.
The following step consists in analyzing digests of proteins with two different mass spectrometers: a quadrupole ESI-Ion Trap (Esquire HCT) coupled to a nano-2D-LC, and a triple quadrupole ESI (Micromass Quattro Ultima) coupled to a LC. The first mass spectrometer is very sensitive to salts, so a last desalting of the sample was done with μC_{18} ZipTips. This step is not necessary with the second mass spectrometer. The sample analysis in 1D-LC-MS/MS with Esquire aims to identify proteins and peptides (singly-, doubly- and triply-charged ions) for each identified protein. Results for soy are under treatment but three allergenic proteins belonging to hazelnut (*Corylus avellana*) have been identified in protein databases (SwissProt, NCBi and MSDB) with the search engine Mascot Daemon (Table 11). The protein showing the best MS intensity (*i.e.*, the highest Mowse score) was selected: *Cor a 9*. It means this protein is easily detectable in LC-MS. Then, among the list of its detected peptides, peptides were eliminated which do not match to criteria 1 to 4 of Table 10 . Thirteen peptides have no misscleavages but one peptide contains a terminal arginine, representing a risk of misscleavage. Ten have no cystein, not any contains variant amino acid and eight are specific to hazelnut (peptide specificity is determined with the protein BLAST program http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Two others peptides are eliminated because their identification is not systematic in replicates. Thus, we obtained six candidate peptides to be potentially the target peptide or the internal standard for the quantification of hazelnut in food (Table 12)

Table 11: Identified proteins (allergens) of hazelnut in 2D-LC-MS/MS on Esquire.

Protein name	Synonyms	Mowse score (MSDB database)
11S globulin-like protein	**allergen *Cor a 9***	**1665**
48kDa glycoprotein	7S vicilin-like protein allergen *Cor a 11*	278
Lipid transfer protein	allergen *Cor a 8*	81

Table 12: Mass-to-charge ratios of four peptides matching to criteria 1 to 4, candidate to be the target peptide or the internal standard for hazelnut quantification with the Heavy peptides method.

Candidate peptide	Mass-to-charge ratio (m/z) of peptides (Da)		
	Singly-charged parent ion	Doubly-charged parent ion	Triply-charged parent ion
[195-208] HFYLAGNPDDEHQR	1697.8	849.9	566.9
[351-363] INTVNSNTLPVLR	1439.8	720.9	480.9
[401-417] VQVVDDNGNTVFDDELR	1933.9	968	645.6
[464-478] **ALPDDVLANAFQISR**	1628.9	**815.5**	544

To test criteria 5 to 7, a second sample analysis was done in 1D-LC-MS/MS with Micromass Quattro Ultima which is the instrument for routine analysis. The source is an electrospray too, so we should find the same peptides than with the Esquire. This kind of mass spectrometer enables to do a full MS scan of the sample (Daughters mode), a MS/MS scan of daughters ions resulting from the fragmentation of peptides (MRM and SRM modes) and to determine fragment ion intensities using the MassLynx software. In MRM mode, several transitions can be monitored in the same time window whereas in SRM mode one transition is monitored. A transition means the fragmentation of a parent ion in a given daughter ion, and is written as a couple «m/z parent ion → daughter ion».
Firstly, a liquid chromatography followed by a daughter scan allowed us to acquire a chromatogram and a full mass spectrum for the four candidate peptides (doubly- and triply-charged form) mentioned in the Table 12. Only the doubly-charged ion m/z 815.5 of the peptide ALPDDVLANAFQISR shows a correct elution (Figure 43). All daughter ions (*i.e.* electrically charged products of reaction of a particular parent ion involving a change in the number of charges carried (Inczédy et al., 1997)) belonging to this candidate peptide and showing a good MS/MS signal are searched on the corresponding spectra (Figure 44) and listed in Table 13.

At least 2 transitions have to be used to perform the quantification of hazelnut allergens in food using the *Heavy Peptides method*. A subsequent MRM experiment, set at different collision energies, allowed us to acquire a mass spectra for each transition mentioned in the Table 13 and to determine at which optimal collision energy the transitions have the highest intensity (Table 13). The intensity of the MS/MS signal of a daughter ion is equivalent to a peak area on the corresponding chromatogram, thus, the abundance of ions can be calculated.

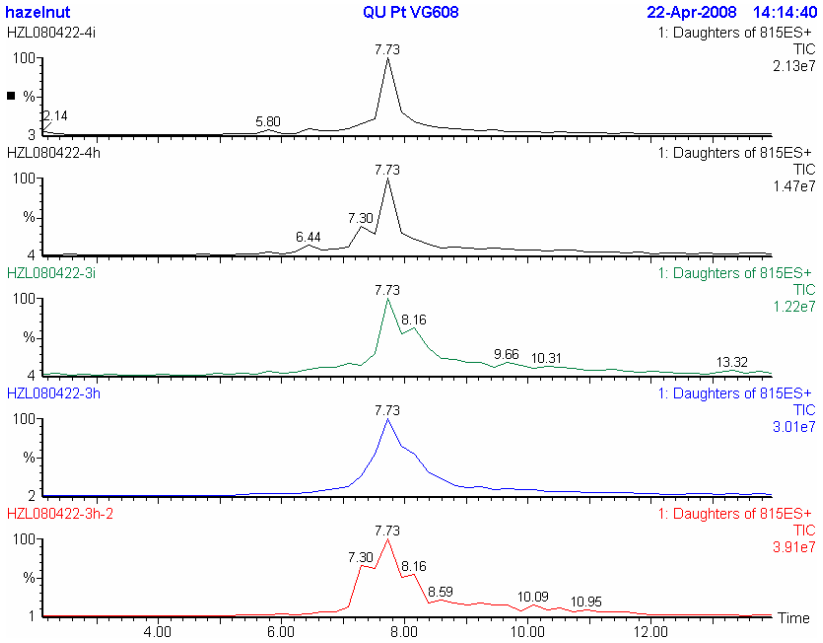


Figure 43: Chromatograms of the doubly-charged parent ion ALPDDVLANAFQISR at different collision energies (10, 20, 25, 30 and 35eV from top to the bottom).

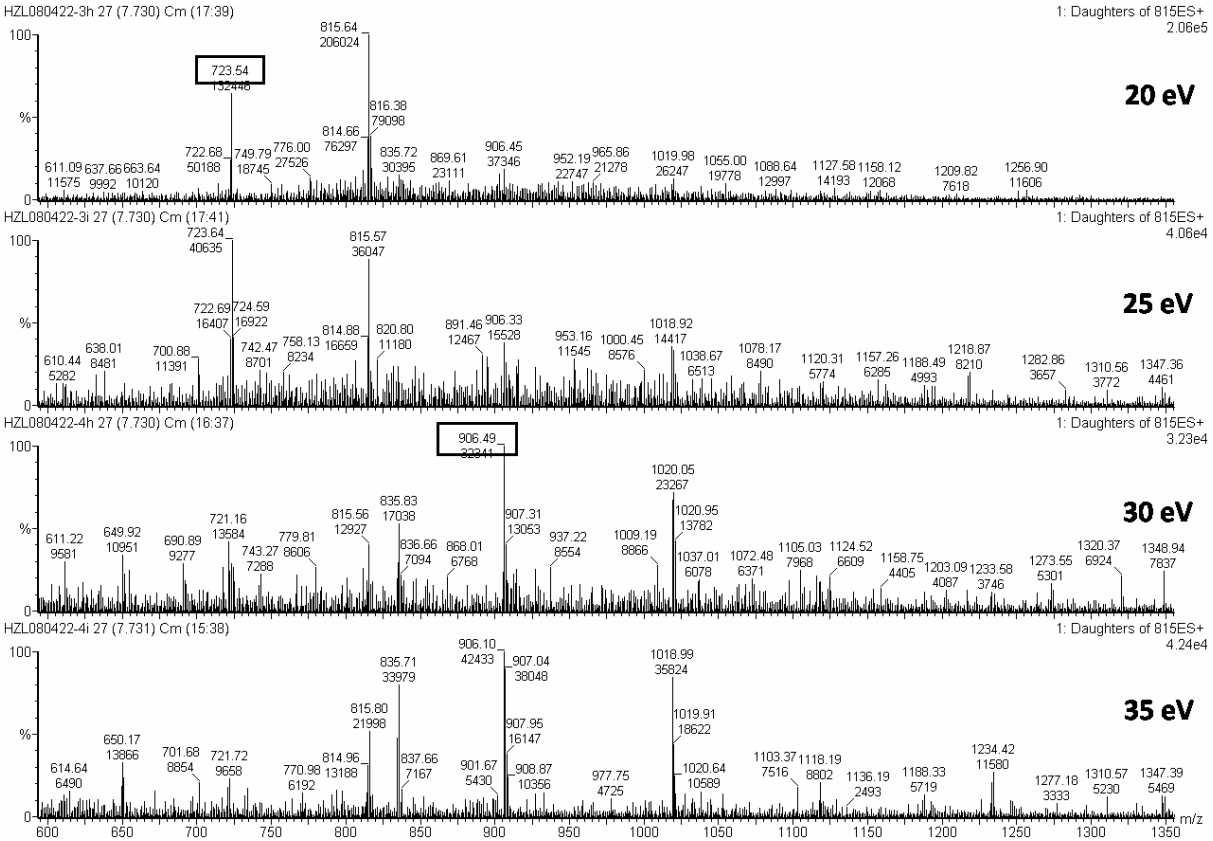


Figure 44: Four daughter scans of the doubly-charged parent ion ALPDDVLANAFQISR m/z 815.5 at four collision energies (20, 25, 30 and 35 eV). The two most intense daughter ions m/z 723.5 and m/z 906.5 are framed in black box.

Table 13: Mass-to-charge ratios (m/z) and optimal collision energy for the 4 candidate daughter ions of the peptide [464-478] ALPDDVLANAFQISR.

Number of the transition	m/z of daughter ions	Collision energy (eV)
1	723.5	20
2	835.8	20
3	906.5	30
4	1019.9	25

According to the criteria 6, transitions have to be specific to avoid erroneous quantification of the corresponding allergen. Specificity of pair of transitions is determined with the PepFrag program available on internet (http://prowl.rockefeller.edu/prowl/pepfrag.html). Transition pairs are tested one by one (1+2; 1+3 and 1+4) with PepFrag but any pair is specific to *Corylus avellana*. Some proteins belonging to the taxon «Viridiplantae» generate peptides with the same m/z in mass spectrometry and the same retention time in liquid chromatography than the peptide ALPDDVLANAFQISR. Transitions triplets are then tested and the combination 1+2+3 is specific. It means that any proteins belonging to a green plant produce similar transitions in LC-MS/MS, what ensures a specific quantification.

To summarize, the target peptide selected is the doubly-charged ion m/z 815.5 of the peptide ALPDDVLANAFQISR. Its three specific transitions which will be monitored in MRM are the following: m/z 815.5 → 723.5, m/z 815.5 → 835.8 and m/z 815.5 → 906.5, optimal at collision energies of 20, 20 and 30 eV respectively.

- ***Selection of the internal standard***

As most of criteria are common for the choice of the target peptide and the internal standard, the same peptide coming from Cor a 9 is selected to be the internal standard for hazelnut. The peptide has been then synthesized with stable ^{13}C and ^{15}N incorporated on the eleventh aminoacid a phenylalanine: **ALPDDVLANA*FQISR**. The mass of this heavy peptide is 1639.9 Da, so the expected m/z ratio for the doubly-charged parent ion is 820.1 Da (calculated according the formula m/z ratio = (m+2)/2 where m is the mass of the singly-charged parent ion). After optimization of MS/MS parameters in direct injection, a liquid chromatography is performed, followed by a daughter scan in order to acquire a chromatogram and a full mass spectrum for the heavy peptide (doubly- and triply-charged form). Only the doubly-charged ion m/z 820.9 of the peptide ALPDDVLANAF(10)QISR shows a correct elution (Figure 45). All daughter ions belonging to the heavy peptide and showing a good MS/MS signal are searched on the corresponding spectra (Figure 46) in order to select at least two specific transitions. The three following transitions are interesting: m/z 820.9 → 728.5, m/z 820.9 → 916.8 and m/z 820.9 → 1030. This transition triplet is finally selected according to the analysis with PepFrag. A subsequent MRM experiment permitted to set the optimal collision energies for the three transitions: 20, 25 and 35 eV respectively.

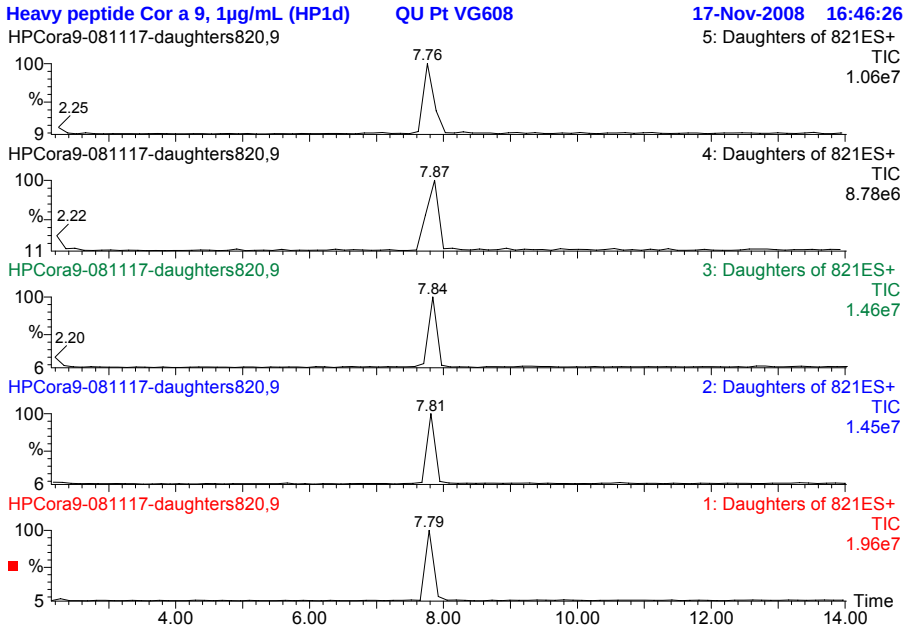


Figure 45: Chromatograms of the doubly-charged parent ion ALPDDVLANAFQISR at different collision energies (15, 20, 25, 30 and 35eV from the bottom to the top).

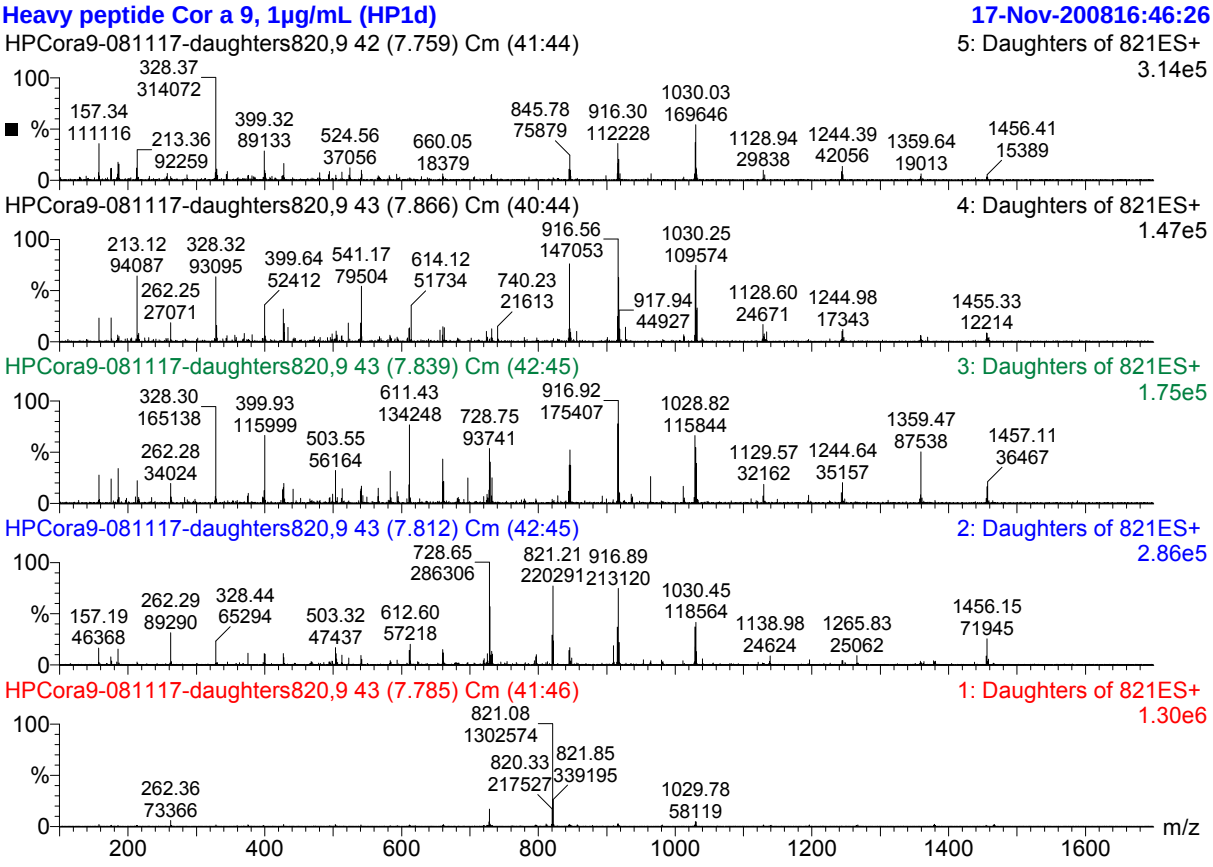


Figure 46: Five daughter scans of the doubly-charged parent ion ALPDDVLANAF(10)QISR m/z 820.9 at five collision energies (15, 20, 25, 30 and 35 eV).

- ***Validation of the internal standard in food matrix***

Once the internal standard is characterized, it has to be validated with the matrix effect. This step is not started yet. A biscuit matrix will be spiked with two different amounts of heavy peptide and the intensity of the signal will be observed with and without matrix. If the intensity is still above 100000, the heavy peptide is validated.

- ***Calibration curve***

As a tryptic digestion is not complete, the intensity of ion (target peptide) measured in MS/MS cannot be directly correlated with the abundance of the protein in the food sample. It is necessary to know which concentration of protein gives which intensity in mass spectrometry. That is why the second part of the development of the Heavy peptides method consists in building a calibration curve with the allergenic material we want to quantify: a total protein extract of the seed (hazelnut or soy) or a purified extract of an allergen of interest.

The calibration curve can be performed with the total protein extract of hazelnut supplied by ILVO in the case of the whole hazelnut quantification, but encounters some problems in the case of the quantification of a hazelnut allergen. Recombinant allergens of purified extract of hazelnut allergens are not commercially available, except *Cor a 1* sold by Biomay Labs. To quantify a particular allergen without purified extract of this latter to set up the calibration curve, an alternative way is to proceed by standard addition. The calibration curve is set up with increasing amounts of food sample (*e.g.* chocolate block with hazelnut). During the SRM experiment, the transitions previously selected for the target peptide belonging to the allergen of interest are monitored. The values of normalized abundances are plotted on a graph. The curve does not pass by the origin but intercepts the x-axis before zero. The distance on the x-axis between the interception and the origin represents the amount of protein of interest originally existing in the food sample. This step is not started yet.

Absolute quantification of allergens in food

The third part of the development of the Heavy peptides method consists in testing the absolute quantification step. A known amount of heavy peptide is added to an unknown protein extract of allergen-containing food. The digestion is performed and the peptide mixture is analysed in LC-MS/MS in SRM mode. The abundance of the target peptide (peak area read on the chromatogram) is normalized by the abundance of the IS and the resulting ratio is reported on the y-axis of the calibration curve, established with the protein corresponding to the target peptide. The corresponding value on the x-axis is the concentration of the protein of interest in the food sample. This step is not started yet.

- Conclusion

To conclude on the progress report of the WP3, we can say that the development of the Heavy peptide method in the field of food allergy is in the experimental phase. The sample preparation has been improved, the target peptide and the internal standard for hazelnut have been chosen, and the internal standard will be soon validated in biscuit matrix on the triple quadrupole. The validation of the mass spectrometric method will be done on a food matrix sample containing an unknown amount of hazelnut allergens. The same work on soy is in progress.

4 Adjustments made to the original planning

For the validation of the ELISA assays, the induction of chemical modifications and the development of a quantitative mass spectrometric method, a total protein extract is needed. For this study to be valuable for the industry as well as for the allergic individuals, it is important that an extract is used in which the allergenic properties are conserved. In other words, the protein extract has to be able to elicit an allergic response. Initially protein extract was prepared according to the method described by Yeung and Collins (Yeung and Collins, 1996), as this method has been successfully used by Bruno De Meulenaer for development of an ELISA to detect peanut (De Meulenaer et al., 2005).

There is no standard protocol available for the protein extraction from allergen-containing foods and a diversity of protocols is described. It was found to be worthwhile to examine protein extracts prepared by different protocols for their biochemical characteristics and functionality. On the one hand, the work needed to obtain a generally useful protein extract caused some postponement of several tasks, like the validation of the ELISA kits and the 2D DIGE analysis of the protein extracts. For the same reason the methods to induce chemical modifications were initially optimised with whey protein, causing delay in the production of Ab's and the development of an ELISA, and the "Heavy Peptide Method" was first developed for milk proteins.

On the other hand, the DNA work that was originally planned for a later stage was already initiated. Optimisation of material pre-treatment and DNA extraction was already performed and the development of new real-time PCR methods initiated, which was planned for the second phase.

Table 14: Planning overview

	Phase 1				Phase 2			
	1-6	7-12	13-18	19-24	25-30	31-36	37-42	43-48
WP1: Evaluation of the robustness of the available methods								
- Functional evaluation of protein extracts								
- Validation ELISA's								
- DNA extraction								
- Validation PCR's								
- Comparison ELISA-PCR								
- Chemical modifications								
- Mass spectrometric study								
- *In vitro* techniques								
WP2: Evaluation of optimised integrated analytical techniques								
- Production Ab's								
- Development ELISA								
- Robustness ELISA								
- Development real-time PCR								
WP3: Development quantitative mass spectrometric methods								
- D2 DIGE								
- Identification peptides								
- Mass spectrometry								
- Validation								
WP4: Evaluation of detection limits by *in vitro* techniques								
WP5: Implementation of the strategy in the food and catering industry								
- Critical control points								
- Relevance HACCP								
- Preventing measures								
- Integration in autocontrol								

8 Perspectives Phase 2

In the second phase the validation of the commercially available ELISA assays will be finished. This includes validation with pure protein extract and chemically modified extract for the soy ELISA kits, assessment of the applicability by analysis of spiked matrix with and without inducing chemical modifications and determination of the specificity for both soy and hazelnut kits. Next to the further development of the real-time PCR assays for the detection of soy and hazelnut, the commercially available PCR kits will be validated with pure DNA extract and spiked matrix with and without inducing chemical modifications. In addition, based on the results of the performance of the chemically modified protein extracts in the different ELISA assays, antibodies will be produced in chickens to develop a new ELISA assay, capable of detecting hazelnut/soy allergens in processed food products. The determined LODs for the different assays will be clinically validated by the BAT. The validation of the different methods will then be completed by comparison of the performance of ELISA and PCR.

The mass spectrometric study will be continued by analysis of soy and hazelnut protein extracts chemically modified through Maillard reaction and lipid-induced oxidation.

The Heavy Peptide Method will be further developed and validated with spiked matrix, with and without induction of chemical modifications.

The final step will be the integration of the developed strategy in the food and catering industry by testing the developed and/or validated methods in practice. Therefore, an attempt will be made to better define the existing Critical Control Points and/or define new Critical Control Points. These will then be tested in practice, for which contacts will be made with industrial partners and a hotel school. This will result in defining preventive measures, which can be implemented in the autocontrol guides to reduce the allergenic risk in the food industry and the catering.

6. Posters - Publications

o Poster presentations + abstract

- ***Comparison of three methods for the isolation of hazelnut proteins.***

Platteau C., Bridts C., Reybroeck W., Devreese B., De Loose M., Daeseleire E., Edo D.
10th International Symposium on Immunological, Chemical and Clinical Problems of Food Allergy.
Parma, Italy, 26-29/05/2008

- ***A new method for absolute quantification in food : the Heavy Peptide method***

Fourdrilis S., Bourgeon C., Kirsch S., Widart J., Maghuin-Rogister G., Scippo M.L., De Pauw E.
10th International Symposium on Immunological, Chemical and Clinical Problems of Food Allergy.
Parma, Italy, 26-29/05/2008

- ***Changes induced in whey protein due to interactions with lipids.***

Cucu T., De Meulenaer B., Kerkaert B., Mestdagh F., Devreese B.
Communications in Agricultural and Applied Biological Sciences, 73, 115-118

o Publications

- ***Comparison and functional evaluation of different extraction methods for the isolation of hazelnut (Corylus avellana) proteins***

Platteau C., Bridts C., De Loose M., Daeseleire E.,Taverniers I., Ebo D.
(In progress)

- ***Quantitative methods for food allergens: a review***

Fourdrilis S., Dobson R., De Pauw E., Kirsch S.
(In progress)

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