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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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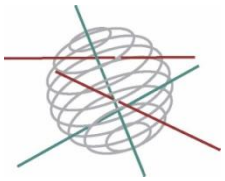

***AGRO-FOOD***

FINAL REPORT

# Development of an integrated strategy for controlling the allergen issue in the Belgian food and catering industry

**ALLERRISK**

**SD/AF/03**


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## SUMMARY

The principal goal of this study was to evaluate different methodologies for allergen detection in food and develop new, improved assays.

In this study we linked the results of the bio-analytical evaluations to the clinical impact for the allergic patient. Therefore, it was important that the bio-analytes used for the studies were clinically relevant, i.e. containing the relevant allergens and in a form being able to provoke an allergic response. Evaluation of different hazelnut protein isolation protocols highlighted the relevance of applying protease-inhibitors during the extraction procedure to preserve the proteins in their native conformation.

The investigated commercial ELISA's showed a lower analytical sensitivity than indicated by the manual. The hazelnut kits were able to detect 1 ppm of defatted hazelnut powder in food model systems, corresponding to about 2,6 ppm hazelnut. The evaluated PCR assays for hazelnut detection showed a positive result for all replicates tested starting from 100 ppm defatted hazelnut flour in the food model systems. The lower sensitivity of the real-time PCR detection platform is probably related to the lower abundance of DNA as target analyte compared to the proteins targeted in ELISA, rather than being inhering to the PCR assay itself. The selected DNA isolation protocol yielded DNA of good quality with respect to the purity and integrity of the DNA. High purity has often a negative impact on the yield. Perhaps the sensitivity of the PCR platform could be increased by applying a DNA isolation protocol returning a higher yield. Our results also confirm that a higher sensitivity is generally associated with a lower specificity, as observed for the commercial ELISA assays for hazelnut and soy evaluated in this project.

Food processing simulating reactions had a severe impact on proteins. As a result of the Maillard reaction, increases in protein bound carbonyls, losses of available lysine residues and severe aggregation was observed. Due to oxidation, either lipid- or hypochlorous acid-induced, modification of essential amino acid was observed linked to an increase in protein bound carbonyls and severe protein aggregation. Further, partial peptic hydrolysis led to almost complete hydrolysis of the hazelnut proteins while the soybean proteins were more stable as evidenced by increases in the free amino groups and the non-protein nitrogen fraction. Despite these severe changes on protein level, several peptides derived from hazelnut and soybean proteins remained stable. Peptides derived from the *Cor a* 9 allergen from hazelnut and the *Gly m* 5 and *Gly m* 6 from soybean were shown to be the most stable as compared to other allergens. These stable peptides could serve as analytical targets for the development of new robust analytical approaches for detection of undeclared allergens in especially highly processed foods.

Further, both the ELISA and real-time PCR detection platform were susceptible to food processing and suffered from interference of the matrix. This was also evidenced by studying the impact of food processing simulation reactions on the detection of hazelnut and soy by commercial ELISA kits, where the observed impact on the detectability was highly dependent on the ELISA kit used. Based on the obtained results it cannot be concluded whether DNA is less susceptible to food processing than proteins, as is often stated. Only a semi-quantitative comparison could be made between both detection platforms, indicating that the impact of food processing was highly variable between the different assays from both types of detection methods.

The commercial ELISA's proved to fail detecting or correctly quantifying the (processed) proteins possibly because the antibodies used for their development are generated against native proteins and their reactivity towards the modified proteins is decreased because the specific epitopes are altered during food processing. Therefore, new ELISA assays were developed using antibodies against modified protein extracts. The newly developed ELISA's showed high specificity towards the modified hazelnut and soybean, with relatively low specificity towards the native ones and without any additional cross reactivity with other nuts or legumes. The robustness of the developed ELISA's was assessed in in-house baked cookies spiked at different levels. Upon spiking of the blank cookies before the extraction, an extensively used approach for the assessment of the robustness of the ELISA, high recoveries were obtained for a wider range of spiking levels. However, upon spiking before baking, low recoveries were obtained probably due to decreased extractability of the antigen from the processed food. A comparison of the performance of the ELISA using antibodies against modified protein extract with a new ELISA against Kunitz trypsin inhibitor (reported stable protein) was made. This showed that using antibodies developed towards allergens modified through food processing simulating reactions is a better approach to be used in food allergen detection.

Target peptides specific of major soy and hazelnut allergens were chosen in the aim of developing a quantitative mass spectrometry-based method to detect trace amounts of allergens in food. For hazelnut, a method for the detection and quantification of 11S globulin-like protein (*Cor a* 9) in food has been developed. Semi-quantification of *Cor a* 9 was carried out in shop-bought chocolate and cookies and in home-made cookies. This is the first mass spectrometric method for the detection and semi quantification for *Cor a* 9. For soy, two calibration curves consisting of either uncooked or baked cookies spiked with increasing amounts of soybean protein extract were constructed. The limit of detection was determined for the baked cookies: 50 ppm soybean protein extract spiked. Two shop-bought samples were analysed: a type of biscuit and a cream dessert. The method worked well for both.

The most important conclusion of this research is that basophils of patients with documented severe hazelnut allergy can be used to demonstrate functional active hazelnut allergens in different matrices. Although this technique cannot be used on a routine base, it might be most valuable, to analyze food containing potentially clinically relevant traces of allergen which might not be demonstrated using "classical" techniques like ELISA or PCR.

At present, the available routine detection methods cannot be applied to assess the remaining allergenicity in food products. Routine assays can only give information on the presence of the food species or particular allergens. As explained above, allergenicity can only be tested with functional assays applying blood from allergic individuals, which makes these tests unfeasible as routine tests. However, it must be noted that the BAT has a very good sensitivity as compared to the other evaluated methods.

This study clarifies that both ELISA and PCR are in theory equally suited for allergen detection in food as they are both only to provide information on the presence of the concerning allergeninc ingredient. Even if an ELISA assay targets the particular allergenic proteins, it cannot serve as an allergy assessment tool, as it merely detects the protein but cannot determine the allergic potential. The assessment of the commercial analytical tools proves that the accuracy of the quantification/detection is undermined by food processing and matrix effects. Performing a risk assessment concerning the impact of food processing on the detection of food allergens by means of routine/commercial tests is complicated, as the results of this study have demonstrated that the impact of chemical modifications as induced by the processing is dependent on the assay used (detection) and moreover varies among sensitised individuals (allergenicity). With regard to studies investigating the impact of food processing it must also be emphasised that extraction of the analyte from the food matrix after processing remains an issue for the different detection platforms (ELISA, PCR, MS, BAT). In this context, MS based detection methods are promising as more harsh extraction procedures can be applied here, as this technique does not analyse intact proteins but detects peptides instead.

To conclude, this study has demonstrated that kit developers should be urged to perform a more extensive evaluation of the currently available routine tests, similarly as has been performed within this project. This information should support the food industry to make an educated selection on the most appropriate test for the intended application.

Key words: food allergy, allergenicity, ELISA, PCR, mass spectrometry, BAT

# 1 INTRODUCTION

The last few years food allergies have become an important issue for both society and the food industry. 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.. The exact prevalence of food allergies is not known, but it is estimated that about 6-8% of children and 2-3% of adults suffer from a food allergy (2001). Although several therapies are being investigated, at present the only effective treatment for food allergy is still 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, processing, shipment, as a result of rework or inadequate cleaning of the food production or preparation equipment.

Directive 2007/68/EC, amending Directive 2000/12/EC, contains a list of 14 allergenic foods, which have to be declared unambiguously on the product label whenever used as an ingredient (European Parliament and Council, 2007). However, the legislation does not cover the presence of unintentionally added allergens as a result of cross-contamination. Nonetheless, the food industry is obliged to cover this item due to their liability to produce safe food for the consumer according to the general Food Law (European Parliament and Council, 2002). 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 and robust to be applied for a broad range of processed food products. The currently available methods detect either the allergenic proteins (e.g. ELISA) or a DNA marker of the allergenic component/product (e.g. PCR) (Poms *et al.*, 2004). In addition, mass-spectrometric methods are being developed to quantify allergens (Monaci and Visconti, 2009b).

The objective of this project is to develop an integrated control strategy for 2 selected allergenic foods, namely soy and hazelnut. This objective comprises the evaluation of currently available detection methods and the development of new detection methods.

The performance of the currently commercially available ELISA- and PCR-based detection kits was evaluated for their analytical and practical applicability. To this end following parameters were evaluated: analytical and practical sensitivity, specificity and robustness with regard to food processing. The impact of the food processing on

the detection was correlated to the induced protein modifications and the impact on the allergenicity to evaluate the possible risk for the allergic consumer.

The development of new analytical assays has been directed towards the detection of hazelnut and soy specifically in processed food products. Those process parameters that result in the highest level of protein modification and showed to be the most critical for the allergen detection with the commercial kits, formed the basis for the development of new improved ELISA methods. As a confirmation method for the above mentioned detection methods a mass-spectrometric method was developed. The applicability of a quantitative technique of analysis based on liquid chromatography coupled to mass spectrometry will be tested on the allergens.

The screening of food products (and their production and processing sites) to detect possible allergens is an essential part of a solid preventive policy. This project results in a comparative evaluation of different detection platforms for food allergens. The final goal is to develop an integrated detection strategy for hazelnut and soy in the food industry. This detection strategy should determine which detection platform/method is best suited for a particular application. The government and producers will also be provided with new tools to control the legislation concerning allergens in a reliable way.

# 2 METHODOLOGY AND RESULTS

## 2.1 Evaluation of protein and DNA isolation protocols

At present there is a lack of standard reference materials for allergens and standard protocols for the isolation of bio-analytes from allergenic foods. In this study a comparative evaluation was performed of different protocols for the isolation of proteins and DNA from allergenic foods. The allergenic foods that have been studied in the framework of this project are hazelnuts and soybeans.

### 2.1.1 Functional evaluation of protein isolation protocols

Besides the fact that a reference material for both concerning allergic commodities is not available, there is a considerable variation among the protein/allergen extraction protocols used with no universal standardised protocol yet being established. From a clinical point of view it is a prerequisite to perform our investigations with a biologically active allergen extract. This means that the allergen extract should be representative for the concerning crop (contain the particular allergens) and be able to induce an immune response in allergic patients. Therefore, a suitable protein extraction protocol fulfilling these requirements is vital. The selection of extraction protocols evaluated in this study was based on existing methods to produce allergen extracts for (food) allergy research. This comparative study was performed on hazelnuts.

Three extraction buffers (A-C), commonly used for the isolation of proteins from allergenic foods, have been compared. The extraction buffers used in the different protocols are;

- Method A: PBS pH 7.4 (1M NaCl, 1.47 mM $KH_2PO_4$, 2.7 mM KCl and 10 mM $Na_2HPO_4$, sodium azide 0.2 g/ L was added to the extraction buffer to avoid microbial growth (Kujala *et al.*, 2002)
- Method B: PBS pH 7.4 , 2% polyvinylpyrrolidone (PVPP) (Björksten *et al.*, 1980)
- Method C: 20 mM $Na_2HPO_4$, 1 M NaCl, pH 7.4 (defatting with hexane) (Yeung and Collins, 1996)

The detailed extraction procedures are described by Platteau *et al*. (2010b). In our investigation we have looked at the influence of the use of a mix of protease inhibitors (2 mM ethylenediaminetetraacetic acid, 5 mM diethyldithiocarbamate, 0.5mM benzamidine hydrochloride and 0.2 mM phenylmethylsulfonefluoride), as described by Björksten *et al*. (1980) on the quality of the protein extract. In Method C the ground hazelnuts were first defatted with hexane before the protein extraction. The impact of the defatting step on the protein extract was also evaluated.

The three extraction protocols were applied with (+) or without (-) the use of the protease inhibitors. Proteins were quantified in the extracts obtained with the DC Protein Assay (Bio-Rad, Hercules, CA, USA). The extracts were first evaluated by loading and separation of equal amounts of proteins of each extract on SDS-PAGE (Figure 1).

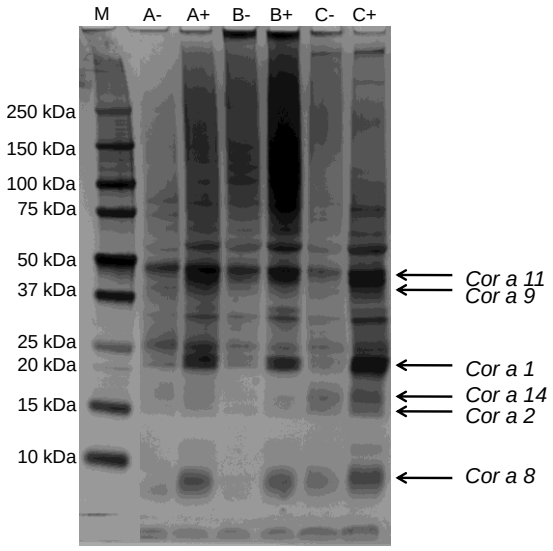


Figure 1 SDS-PAGE of extracts A, B and C, prepared without (-) and with (+) the mix of protease inhibitors (M= molecular weight marker)

Although the amount of proteins that was loaded onto the gel was identical for the different extracts, the intensity of particular bands differs visually between extracts prepared with and without the protease inhibitors. For the extracts prepared with protease inhibitors (A+, B+ and C+) some bands are more intense compared to the equivalent protein bands in the extracts where no protease inhibitors were included during the extraction (A-, B- and C-). It is thus worthwhile to test the functionality of the protein extracts to elicit allergic reactions.

The functionality of the extracts was analysed in the Basophil Activation Test (BAT). The BAT is a flow cytometric method that closely resembles the *in vivo* pathway leading to allergic symptoms (Ebo *et al.*, 2008). Upon activation of basophils, mediators are released, such as histamine, that are responsible for the allergic response. For the test peripheral blood basophils from three hazelnut-allergic patients were applied and the analysis was performed as has been described (Ebo *et al.*, 2007; Platteau *et al.*, 2010b).

Figure 2 shows a dose-response curve of hazelnut extracts A and B prepared without (left) and with protease inhibitors (right). From Figure 2 it appears that adding enzyme inhibitors enhances the allergenicity of the hazelnut proteins in the extracts. Actually, upon stimulation with 1 and 10 µg/mL hazelnut proteins, a lower percentage of activated (CD63+) basophils was measured for two out of three patients for extract A (Figure 2, triangles and circles) and B (Figure 2, triangles and squares), when no protease inhibitors were included. Overall, for extract A the protein concentration

needed to activate the basophils differs 10-fold when prepared with or without protease inhibitors, respectively. For the third patient a strong activation was already observed with extracts A- and B-, demonstrating the high sensitivity of this patient. These results demonstrate the importance of adding protease inhibitors during the isolation of proteins from food.

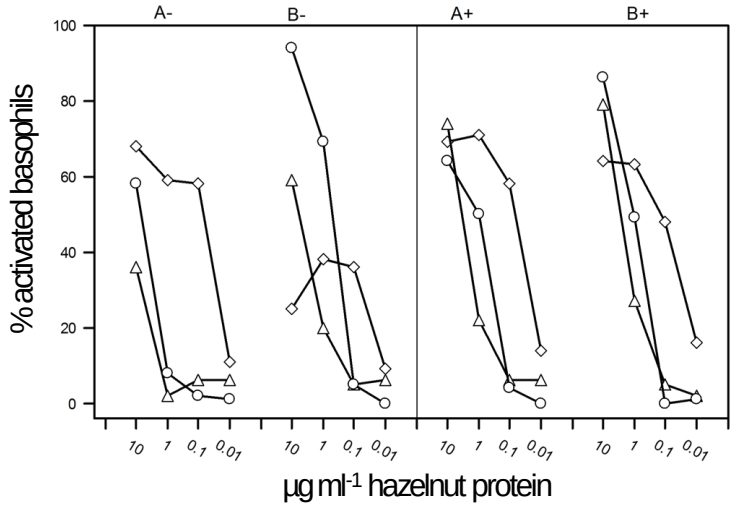


Figure 2 Dose-response curve in BAT of extracts A and B, prepared without (-) and with (+) protease inhibitors, with blood from three hazelnut-allergic patients (triangles, squares, circles).

To investigate the impact of defatting on the hazelnut protein extract, extract C+, was compared to extract A+ and B+ in the BAT. Defatting is often performed to ameliorate the protein extraction.

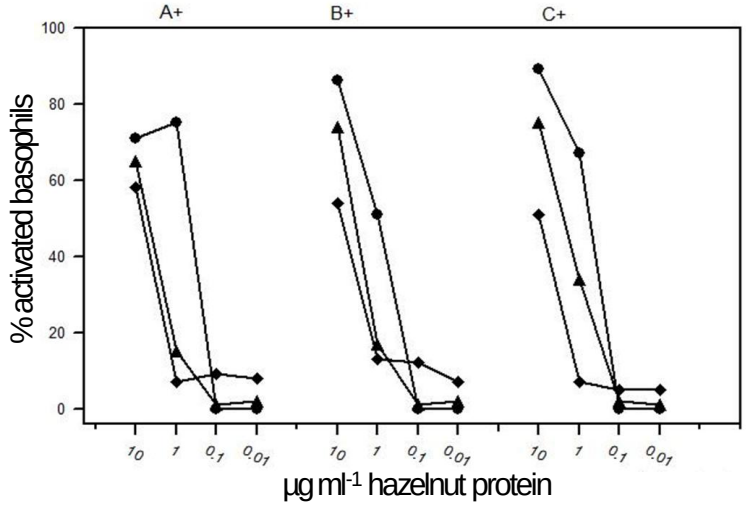


Figure 3 Dose-response curve in BAT of protein extracts A, B, C of hazelnut prepared with protease inhibitors (+) with blood from three hazelnut-allergic patients (triangles, squares, circles).

Figure 3 shows that the percentage of activated (CD63+) basophils differs among the three patients, indicating diversity in allergic response between the patients. However, stimulation with the three extracts results in a similar activation pattern of basophils for each individual patient. From this we can conclude that defatting the

hazelnuts before extraction does not affect the allergy-provoking potential of the extracts for the selected patients. Moreover, it can be concluded that all three extraction protocols (A+, B+ and C+) are suitable for the extraction of proteins/allergens from hazelnuts. The BAT as presented in Figure 3 has also been performed with protein extracts from soybean obtained with the same protocols (A+, B+, C+) applying blood from soy-allergic patients (Figure 4).

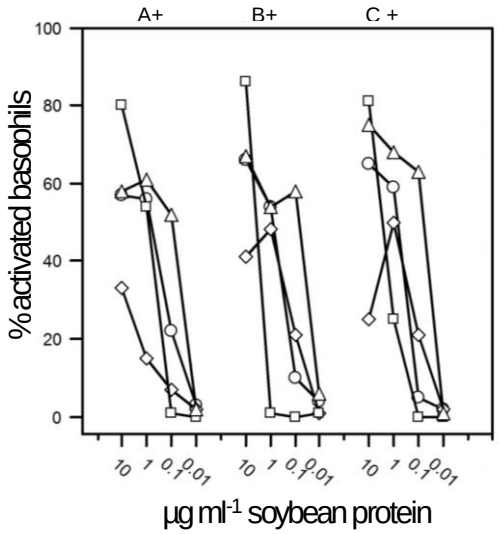


Figure 4 Dose-response curve in BAT of protein extracts A, B, C of soybean prepared with protease inhibitors (+) with blood from three soy-allergic patients (triangles, squares, circles).

Similar activation pattern with the three soybean protein extracts was obtained for the three patients. This indicates that the extraction protocols are also equally suited for the isolation of proteins from soybeans. From the three protocols we have selected method C+, including the protease inhibitors and the defatting step, for the further experimental work.

### 2.1.2 Evaluation of DNA isolation protocols

Further on in this work the development of new PCR assays for the detection of hazelnut and soy is described (2.4.1). These assays were validated together with commercially available PCR assays (Table II) for the detection of both plant species in food. The new assays were developed to be used in a quantitative manner. Although the commercial assays were prescribed to be qualitative, we determined their ability to be applied as quantitative tools. To this end, genomic DNA of both crops was required to be used as a calibrant. The quality of the DNA will be determined by the extraction protocol that is used. The different parameters that define the quality of extracted DNA are the yield, the chemical purity and the structural integrity. These are the parameters that were determined in the evaluation of the different investigated DNA extraction protocols.

The evaluated DNA extraction protocols comprised two CTAB-based protocols, the first one according to De Neve *et al.* (1997) and the second as described by Doyle and Doyle (1990), and three commercial DNA extraction kits: DNeasy Plant mini kit (Qiagen, Hilden, Germany), Wizard® Magnetic DNA Purification System for Food (Promega Benelux, Leiden, The Netherlands) and Nucleospin Food (Machery-Nagel, Düren, Germany).

The first parameter that was determined was the **DNA yield**. To this end the DNA concentration in the different extracts was determined with the Quantit™ PicoGreen® dsDNA Assay kit (Invitrogen Ltd, Paisley, UK) according to the kit instructions. The principle of this assay is based on the binding of the fluorescently labelled dye Picogreen to double stranded DNA. Most studies compare different DNA extraction protocols based on the DNA concentration in the extract. Here the amount of starting material and the elution volume was taken into account to make a correct comparison of the yield (total amount of DNA/amount of start material) obtained with the different methods.The DNA yield obtained after extraction from hazelnuts was clearly lower than from soybean (Figure 5, left). Hazelnuts contain more lipids than soybeans (on average 60 % vs. 20 %) and are consequently more difficult to handle during the extraction procedure. This could explain why less DNA was obtained, although it is also possible that the overall DNA content is lower for hazelnuts. For hazelnut the lowest yield was obtained with the CTAB 1 protocol and the Wizard Magnetic kit. For soybean both CTAB-based protocols showed very poor DNA recovery, although the yield with the CTAB 1 protocol was significantly lower than with the CTAB 2 protocol. For both crops the highest yield was obtained with the Qiagen DNeasy Plant and the Nucleospin kit.

Figure 5 demonstrates that evaluating the yield based on the DNA concentration in the extracts can lead to false conclusions. While the soybean DNA concentration in the extract obtained with the Wizard Magnetic purification and Nucleospin kit was higher than with the Qiagen DNeasy Plant mini kit, the opposite was true for the yield obtained with these kits. This indicates that the Qiagen kit is able to extract the most DNA from soybean.

The low DNA concentrations measured with the CTAB-based protocols could however be explained by a quenching effect of the fluorescence during the measurement by traces of CTAB, as has been published recently, after this investigation was already completed (Holden *et al.*, 2009).

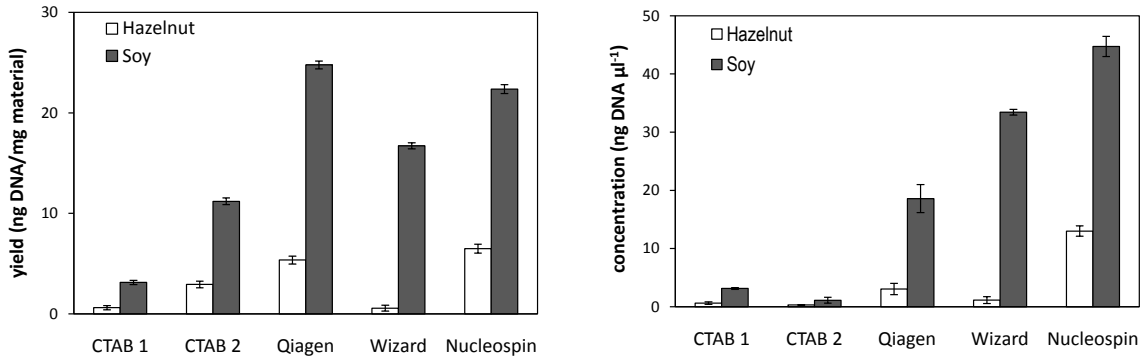


Figure 5 (Left) DNA yield after extraction of hazelnut and soybean using different protocols, (Right) DNA concentration in the different extracts (n=2)

Degradation of the DNA during the extraction process can result from the release of nucleases upon cell lysis. This can be overcome by the addition of proteinase K during extraction, which breaks down the nucleases. In the protocols examined in this study, the addition of proteinase K is specifically described only for the Nucleospin Food kit. The **integrity** of the extracted DNA, the second parameter, was checked by a degradation PCR test. The principle of this test is that several fragments of a conserved eukaryotic 18S ribosomal DNA sequence with a increasing length are amplified by conventional PCR using one common forward primer but with eight different reverse primers, resulting in amplicons with a length ranging from 103 bp to 1468 bp. The length of the longest fragment that is amplified is a measure for the integrity of the DNA (De Latter, 2008). Both hazelnut and soy DNA extracts were analysed with this test. However, amplification was observed only for the soy samples, except for the soy extracts obtained by the CTAB-based protocol according to Doyle and Doyle (Figure 6). This was also the protocol returning the lowest DNA concentration for soy. With the other DNA extracts, amplification of all individual DNA fragments was obtained, showing that the DNA was intact over a length of at least 1486 bp.

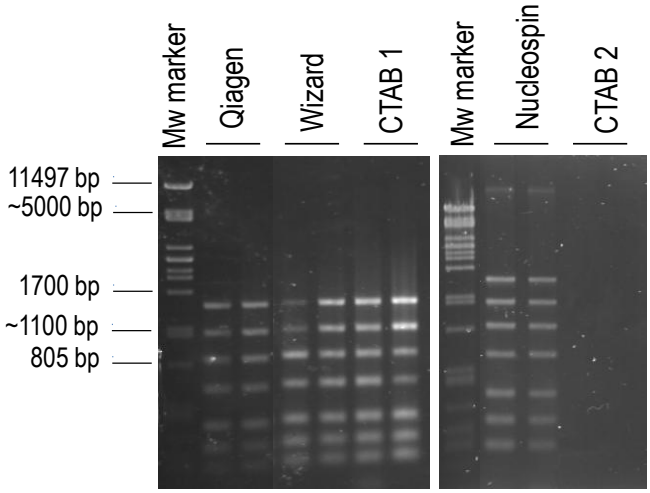


Figure 6 DNA degradation test with different soybean extracts

It was assumed that the lack of amplification for the hazelnut extracts could also be caused by the low DNA yields obtained. In a search to try to increase the DNA yield from hazelnut, a slight adaptation was made to the Qiagen protocol; instead of transferring the clear supernatant of the lysis mixture to the Qiashredder columns, which are intended to remove residual cell debris from the solution, the whole lysate was applied. This proved to increase the DNA concentration in the extracts for both hazelnut and soy from 3.4±1.3 to 12.4±1.5 and 18.4±2.2 to 24.3±4.2 ng $\mu l^{-1}$, respectively. Upon applying these DNA extracts in the 18S degradation test, amplification up to 1486 bp was demonstrated for both hazelnut and soy. As this is the only protocol using this type of 'cleaning' columns, this adaptation was only possible for the Qiagen kit. It was therefore decided to apply this protocol for all further DNA extractions that had to be executed.

**Contaminants** in the DNA extract can impair an efficient amplification of the target DNA sequence. This could lead to false positive results or erroneous quantification of the target analyte. The efficiency of a PCR assay can be assessed in real-time format by constructing a standard curve through analysing a dilution series of the genomic DNA. Under normal conditions a linear relationship exists between the natural logarithm of the template concentration present in the reaction vial and the $C_q$ value obtained after processing the obtained real-time fluorescent signals ($\log(\Delta conc) = (\Delta Cq) \,/\, 3.32$). Deviations from the linear curve indicate an altered PCR efficiency, which can be an indication of the presence of PCR inhibitors in the sample. A dilution series of genomic DNA obtained from hazelnut and soybean with the Qiagen DNeasy Plant mini kit, the adapted protocol as described above, was analysed in a real-time PCR targeting a conserved sequence on the 18S ribosomal DNA.

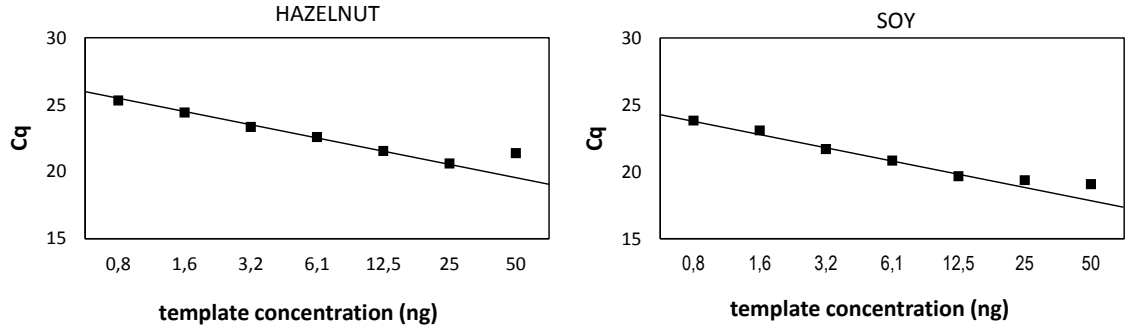


Figure 7 Calibration curve of the 18S inhibition PCR assay with hazelnut and soy DNA

The calibration curves obtained for the universal 18S PCR assay (Figure 7) show deviating $C_q$ values at concentrations above 25 ng and 12.5 ng template DNA for hazelnut and soy, respectively. The $C_q$ values for the samples with higher concentrations are higher than should be expected based on the standard curve, indicating that the amplification reaction was impaired. The decreased efficiency of

the PCR at higher concentrations could be explained by the higher concentration of inhibitory components in the less diluted samples. Further on in this work the linearity of new developed PCR assays for the detection of hazelnut and soy was determined (2.4.1). In contrast to the observations presented here, good linearity between $C_q$ values en template concentrations was observed up to 50 ng total DNA in the PCR reaction for both crops. A possible explanation for this difference could be found in the genome copy number of the target that is amplified. In the case of the 18S ribosomal DNA sequence it is known that the amplified sequence is present in multiple copies on the eukaryotic genome, while the hazelnut and soybean allergen encoding sequences are assumed to be single-copy targets. Amplification of a multiple copy target could lead to competition for the building blocks (nucleotides) and polymerase in the PCR reaction if the template is present at high concentration, leading to a sub-optimal amplification. In this case competition instead of inhibition is thought to be the reason for the decreased efficiency at higher template concentrations. From these findings we can conclude that the decreased PCR efficiency at higher DNA concentrations is not due to the presence of inhibitors, but can be attributed to competition for the reagents in the reaction. In summary, the DNA obtained with the Qiagen DNeasy Plant mini kit is of sufficient purity to be efficiently amplified in PCR. The DNeasy Plant mini kit, with the slight adaptation of the protocol as described above, is therefore used for all further DNA extractions in the project.

## 2.2 Evaluation of the analytical performance and robustness of ELISA and PCR

In this study one of the goals was to evaluate the performance of commercially available ELISA and PCR methods for the detection of hazelnut and soy. The commercial assays that have been included in this work are summarised in Table I and Table II. Some new quantitative real-time PCR assays for the detection of hazelnut and soybean with regard to allergen detection were also developed within the framework of this project (see 2.4.1), as such assays were lacking at the take-off of the project.

Table I Overview of commercial ELISA kits applied in this study

| Allergenic food | kit | LOD | LOQ | Manufacturer |
|---|---|---|---|---|
| Hazelnut | • Veratox | - | 2,5 ppm | Neogen |
| | • Ridascreen FAST | 1,5 ppm | 2,5 ppm | R-Biopharm |
| | • Residue | - | 0,5 ppm | ELISA Systems |
| | • BioKits | 0,1 ppm | 1 ppm | Tepnel (now Neogen) |
| Soybean | • Veratox | - | 10 ppm | Neogen |
| | • Residue | - | 1 ppm | ELISA Systems |
| | • BioKits | 0,3 ppm | 1,25 ppm | Tepnel (now Neogen) |

Table II Overview of commercial real-time PCR kits applied in this study

| Allergenic food | kit | LOD | Manufacturer |
|---|---|---|---|
| Hazelnut | • Surefood Allergen real-time PCR | < 10 genome copies ≈ 10 ppm | Congen |
| | • First PCR Kit | 5 genome copies | Gen-Ial |
| Soybean | • Surefood Allergen real-time PCR | < 10 genome copies ≈ 10 ppm | Congen |
| | • First PCR Kit | 5 genome copies | Gen-Ial |

### 2.2.1 Evaluation of commercial ELISA kits

#### Sensitivity

Allergen detection methods need to be able to detect traces of the allergenic product. The enzyme-linked immunosorbent assay (ELISA) is the preferred method to assess the level of allergens contamination in foods and is extensively used both by the food industry and the enforcement agencies. There is a general agreement that allergen detection methods should have a detection limit between 1 and 100 ppm (Poms *et al.*, 2004), although nowadays a maximum detection limit of 10-20 ppm is preferred. The sensitivity of the ELISA assays was evaluated by determination of the Limit Of Detection (LOD) and the Limit Of Quantification (LOQ). The LOD was determined by analysing 20 blank samples, calculating the mean quantified value and adding three times the standard deviation. For the LOQ 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. The obtained absorbance values for each sample were quantified by means of a standard curve that was constructed with the standard protein solutions included in each kit. For fitting the absorbance values to the protein

concentration of the standard solutions, a four-parameter-logistic dose-response curve was used with equation (1) below. This equation represents a standard way to express immunoassay data, where *a* is the maximum of the curve, i.e. the absorbance at the highest dose (protein concentration), *b* is the minimum, i.e. absorbance at the lowest concentration, *c* is the $E_{50}$-value (concentration at half-maximal saturation), *d* is the slope of the curve and *conc* is the concentration of the standard or sample (Englebienne, 2000).

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

Conversion of the LOD and LOQ value expressed as ppm hazelnut/soybean to the corresponding protein concentration expressed as ng/ml protein was performed according to equation (2) and (3) (see 2.2.3) and is explained below.

Table III LOD and LOQ of commercial hazelnut ELISA kits determined by analysing 20 blank samples (LOD=mean+3SD, LOQ=mean+6SD)

| | | Veratox HN | Ridascreen FAST HN | HN Residue | BioKits HN |
|---|---|---|---|---|---|
| **LOD** | **ppm HN** | 0.058 | 0.014 | 0.0025 | 0.023 |
| | **ng/ml HN protein** | 0.278 | 0.06 | 0.0299 | 0.138 |
| **LOQ** | **ppm HN** | 0.123 | 0.044 | 0.011 | 0.068 |
| | **ng/ml HN protein** | 0.59 | 0199 | 0.1264 | 0.409 |

Table IV LOD and LOQ of commercial soy ELISA kits determined by analysing 20 blank samples (LOD=mean+3SD, LOQ=mean+6SD)

| | | Veratox Soy | Soy Residue | BioKits Soy |
|---|---|---|---|---|
| **LOD** | **ppm soy** | 1.582 | 0.136 | 0.695 |
| | **ng/ml soy protein** | 63.29 | 13.58 | 6.946 |
| **LOQ** | **ppm soy** | 2.565 | 0.579 | 1.319 |
| | **ng/ml soy protein** | 106.24 | 57.94 | 13.18 |

The determined LOD and LOQ values of the commercial ELISA kits for the detection of hazelnut and soy (Table III and Table IV) are much lower than the values specified by the kit manufacturer (Table I). It could be that the latter used another method to determine these values, although, determination of the LOD for the hazelnut kits with a dilution series of hazelnut protein, prepared by the selected method described under 2.1.1, returned similar values as in Table III (data not

shown). This indicates that these kits have a better analytical sensitivity than specified by the manual. However, it must be kept in mind that the analytical sensitivity (detection limit of the analyte as such) is not necessarily equal to the practical sensitivity (detection limit of the analyte contained in a food product). The latter was also investigated in this project (see section 2.2.3).

**Specificity**

The examined ELISA assays are intended to analyse food products containing a broad variety of ingredients. Hence, it is important that the assays will only detect the intended target and will not cross-react with other possible ingredients present in the food product, which could lead to a false positive result. Cross-reactivity can occur between homologous proteins having a similar structure or amino acid sequence. As it is practically impossible to test all existing foods for possible cross-reactivity in the ELISA assays, a selection was made. The selection was based on (a) the results of a Protein BLAST of the known hazelnut and soy allergens to search for other food species containing homologous proteins, (b) the results of a Primer BLAST of our own developed primers (see 2.4.1) to search for other food species containing a DNA sequence within their genome with which the primers could hybridise, (c) possible cross-reactivity of bulk ingredients present in the food product. In this project cookies were chosen as a model food system. Consequently, the bulk ingredients that were selected are products that can be present as bulk ingredients in cookies.

Proteins were extracted from the selected food products according to the procedure described in the kits manual and analysed according to the manufacturer's instructions.

Table V shows that a lot of the food samples return a positive result in one or more ELISA kits, which is worrisome with regard to false-positive test results of food products containing these ingredients. This could lead to unnecessary product recall, which is harmful for the food producer. . However, not all signals are of the same order. In order to interpret the obtained data, the LOD of the kits, if specified, should be considered as well (values > LOD indicated in black and bold). Since not all of the evaluated kits specify their LOD, however, the experimentally determined LOD was also considered (values > experimental LOD indicated in green). Foods returning values higher than the LOD of the kit as specified by the manual can be considered as true cross-reacting species. Both the BioKits Hazelnut and BioKits Soya assays had the highest number of cross-reacting species. The BioKits Hazelnut assay cross-reacted with multiple other nuts (almond, cashew, macademia, pecan nut, pistachio and walnut) and peanut. However, only cross-reactivity with walnut and pecan is reported by the kit's manual. This can be problematic when a food product containing other nuts than hazelnut is analysed with this kit. Some of these nuts even produce a signal that is higher than the LOQ (red bold) (cashew, pecan nut and walnut). The

BioKits Hazelnut is also the only hazelnut ELISA that returns rather high absorbance values in general. For the Ridascreen FAST Hazelnut kit, only pistachio returns a value above the LOD specified by the kit. For the two other kits, Veratox Hazelnut and Hazelnut Residue, no LOD is specified by the kit. The number of foods producing a signal above the experimental LOD is very high for the Veratox Hazelnut kit. However, the foods returning the highest values are the nuts, peanut and lettuce.

Table V Amount of hazelnut/soy determined with the different commercial ELISA kits in the food products selected for the specificity assessment expressed in parts per million (ppm). Data are mean values of duplicate ELISA analyses of a single sample

| | Veratox for HN | Ridascr. FAST | HN Residue | BioKits HN | Veratox Soy | Soy Residue | BioKits Soy |
|---|---|---|---|---|---|---|---|
| **Almond**[a] | 0,26 | 0,37 | 0,001 | **0,52** | / | 0,09 | **0,59** |
| **Barley**[a] | 0,04 | / | / | 0,02 | 1,80 | 0,13 | 0,05 |
| **Brazil nut**[a] | 0,14 | / | 0,01 | 0,06 | **55,28** | **1,89** | **2,74** |
| **Cashew**[a] | 0,58 | 0,79 | / | **1,60** | 0,97 | 0,13 | **0,55** |
| **Coleseed**[a] | 0,17 | 0,63 | 0,02 | **0,63** | 0,97 | 0,08 | **0,54** |
| **Hazelnut**[a] | - | - | - | | / | 0,07 | **0,81** |
| **Macademia**[a] | 0,14 | 0,01 | 0,001 | **0,45** | / | 0,07 | / |
| **Olive**[a] | / | / | / | 0,08 | 0,51 | 0,13 | / |
| **Pea**[a] | 0,04 | / | / | **0,37** | 0,69 | 0,12 | **4,69** |
| **Peanut**[a] | 0,15 | / | / | **0,26** | 0,96 | 0,05 | **0,72** |
| **Pecan nut**[a] | 0,34 | / | / | **2,22** | / | 0,02 | / |
| **Pistachio**[a] | 0,88 | **2,29** | 0,14 | **1,32** | 0,87 | 0,09 | **0,34** |
| **Soy**[a] | 0,13 | / | / | 0,83 | - | - | - |
| **Wheat**[a] | 0,06 | 0,70 | / | / | 0,90 | 0,05 | **0,79** |
| **Lettuce**[b] | 0,53 | 0,02 | 0,02 | **1,61** | 1,82 | 0,12 | / |
| **Raspberry**[b] | 0,082 | 0,01 | / | / | 0,15 | 0,16 | / |
| **Spinache**[b] | / | 0,09 | / | **0,20** | 0,25 | 0,05 | / |
| **Strawberry**[b] | 0,08 | / | / | **1,33** | 2,25 | 0,10 | / |
| **Apple**[c] | 0,11 | / | / | / | / | 0,03 | / |
| **Grape**[c] | / | / | / | / | 0,83 | 0,16 | / |
| **Maize**[c] | 0,08 | 1,35 | / | / | 1,24 | 0,10 | / |
| **Oat**[c] | 0,09 | 0,16 | / | / | **69,10** | **3,30** | **10,76** |
| **Rice**[c] | 0,17 | / | 0,12 | **0,33** | 3,64 | 0,28 | / |
| **Walnut**[c] | 0,31 | 0,01 | 0,02 | **5,41** | / | 0,04 | / |
| **Egg white**[d] | 0,13 | / | / | / | / | / | / |
| **Egg yolk**[d] | 0,04 | / | / | / | / | 0,09 | / |
| **Kamut**[d] | 0,06 | 0,01 | / | / | **114,86** | **2,70** | **7,19** |
| **Milk (powder)** | / | / | / | / | / | / | / |
| **Spelt**[d] | 0,09 | 0,17 | / | / | **22,91** | **1,05** | / |
| **Starch (potato)** [d] | 0,04 | / | / | / | 0,90 | 0,03 | / |
| **Sugar**[d] | / | / | / | / | / | 0,030 | / |

[a] food selected based on results of Protein BLAST

[b] food selected based on results of Primer BLAST

[c] food selected based on results of Protein and Primer BLAST

[d] bulk ingredient

/ measured absorbance < blank absorbance

value > own determined LOD

**value > LOD specified by kit**

**value > LOQ specified by kit**

For the three soy ELISA kits cross-reactivity can be observed for Brazil nut, oat and kamut. Spelt shows cross-reactivity in two out of three kits (Veratox Soy and Soy Residue). Besides cross-reactivity with multiple nut species (almond, Brazil nut, cashew, hazelnut and pecan), the BioKits Soy assay also shows cross-reactivity with other legumes (pea and peanut), coleseed and wheat. Cross-reactivity of the latter with Brazil nut and different kinds of pea were indicated by the kit. However, the cross-reactivity with walnut could not be confirmed in our study.

It must be emphasised that these results are obtained by analysis of the pure food product, but even then, the obtained values are in general rather low. These foods will not be present at a 100 weight percentage in a food product when included as an ingredient and would then probably not pose a problem. Although, the cross-reactivity observed for kamut and spelt in the soy ELISA kits can be more problematic if these products are used as bulk ingredients, i.e. at a high weight percentage in the food product.

### 2.2.2 Evaluation of new and commercial real-time PCR assays

#### Sensitivity

The analytical sensitivity of both the new and the commercial PCR assays was determined by analysing dilution series of genomic hazelnut or soy DNA, obtained with the DNeasy Plant mini kit (adapted protocol), ranging from 0.256 pg to 50 ng (total DNA content in the PCR reaction). The commercial PCR assays were applied as described by the manufacturer. The newly developed assays are described further on in this work (2.4.1). All assays were real-time PCR tests based on TaqMan detection chemistry. PCR reactions were performed in an ABI Prism 7000 (Applied Biosystems). Data were analysed with the 7000 System Sequence Detection Software Version 1.2.3 (Applied Biosystems). Six replicates of each concentration were analysed. The detection limit (LOD) was defined as the concentration at which all replicates showed an amplification curve and a signal above the threshold was obtained. The LOD was 3.2 pg genomic hazelnut DNA for the *Cor a* 1 and *Cor a* 8 assays and 1.28 pg genomic soy DNA for the *Gly m* Bd 28K and *Gly m* Bd 30K assays (Table VI). The commercial assays for both hazelnut and soy were all able to detect 1.28 pg genomic hazelnut/soy DNA. When converting this amount to the number of copies of the target that can be detected, taking into account the genome size of hazelnut (0.48 pg) (Bennett and Smith, 1976) and soy (1.13 pg) (Bennett and Leitch, 1997), Table VI shows that the commercial kits for soy detection are the most sensitive, being able to detect as less as 1.1 genome copy. However, we must remark that for this calculation it is assumed that the targeted sequences are single copy genes.

Table VI LOD and LOQ of the different hazelnut and soy PCR assays

| **Assay** | **LOD** | | **LOQ** | |
|---|---|---|---|---|
| | ***pg DNA*** | ***copies*** | ***pg DNA*** | ***copies*** |
| • *Cor a 1* | 3,2 | 6,6 | 6,4 | 13,3 |
| • *Cor a 8* | 3,2 | 6,6 | 6,4 | 13,3 |
| • *First Hazelnut* | 1,28 | 2,7 | 6,4 | 13,3 |
| • *Surefood Hazelnut* | 1,28 | 2,7 | 6,4 | 13,3 |
| • *Gly m Bd 28 K* | 3,2 | 2,8 | 3,2 | 5,7 |
| • *Gly m Bd 30 K* | 3,2 | 2,8 | 3,2 | 5,7 |
| • *Surefood Soy* | 1,28 | 1,1 | 1,28 | 5,7 |
| • *First Soy* | 1,28 | 1,1 | 1,28 | 5,7 |

The quantification limit was determined by constructing a standard curve for each assay. The Limit of Quantification (LOQ) was set at the lowest concentration at which a linear relationship exists between the concentration and the quantification cycle ($C_q$) ( $\log(\Delta conc) = (\Delta Cq) / 3.32$). As to date no requirements are available for PCR assays detecting food allergens, evaluation of the calibration curves was done based on the Minimum Requirements of analytical methods for GMO testing (European Network of GMO Laboratories (ENGL), 2008). This document prescribes that the correlation coefficient ($R^2$) has to be ≥ 0.98 and that the slope of the curve between -3.1 and -3.6, resulting in an PCR efficiency of 110 % and 90 %, respectively according to equation following equation: $Efficiency = 10^{\frac{-1}{slope}} - 1$. Each assay evaluated fulfilled these requirements. For each assay the lowest point of the standard curve, i.e. the quantification limit, was 6.4 pg DNA.

**Specificity**

it is important that the assays will only amplify and consequently detect the intended target and will not cross-react with DNA sequences from other possible ingredients present. The specificity of the designed assays was first determined *in silico* by searching for homologies with DNA sequences present in the NCBI database. We did not search for entries containing a nucleotide sequence similar to the whole PCR product, as cross-reactivity could result from the primers binding to another DNA sequence than the target of interest with a differing inter-primer sequence. Instead, a search for sequences which the primers could anneal with was performed using the NCBI Primer BLAST tool. This approach was not possible for the commercial assays as the primer sequences are not released by the kits manufacturers. The *Cor a* 8-primers demonstrated homologies with nucleotide sequences within the genome of apple (*Malus domesticus*), lettuce (*Lactuca sativa*), oat (*Avena sativa*), raspberry (*Rubus idaeus*), strawberry (*Fragaria ananassa*), walnut (*Juglans regia*), grape (*Vitis vinifera*) and soy (*Glycine max*). Although the

alignment of the primers with these sequences contained single or multiple mismatches, successful annealing cannot be ruled out. Primer pairs developed to amplify a fragment of the *Cor a 9* and *Cor a 10* genes of hazelnut showed *in silico* cross-reactivity with spinache (*Spinacia oleracea*) and maize (*Zea mays*) and rice (*Oryza sativa*), respectively. Even though these primers were not used further on this study, these plant species were also included in the specificity test if not yet included based on the protein BLAST selection.

DNA was extracted from all the foods with the DNeasy Plant mini kit (adapted protocol). As a control to avoid false-negative PCR results, the presence of amplifiable DNA was tested in a real-time PCR assay with universal primers and a 5'-FAM-labeled, 3'-TAMRA-quenched oligonucleotide hydrolysis probe targeting a conserved eukaryotic sequence on the 18S rRNA gene (De Latter, 2008). Successful amplification was achieved for all samples. Upon analysis of the DNA samples from the different food species with the different hazelnut PCR assays, positive results were obtained only with the commercial assays for pistachio and lettuce with the First Hazelnut kit and for raspberry and strawberry for both the First and Surefood Hazelnut assay (Table VII). To ascertain that the signal is resulting from cross-reactivity and not originating from cross-contamination of the sample, the results were further investigated.

The most logical approach to elucidate whether or not the food samples are contaminated with hazelnut would be identification of the amplified fragment by determination of the melting temperature ($T_m$) or fragment sequencing. However, these approaches are not possible with the commercial PCR tests, as the identity of the primers is unknown. Also, the primers are contained in a mix with other PCR components in the kits, but the exact composition of the mixes is not known. Therefore, further investigation of these samples was performed by an alternative approach. To this end the samples were analysed in an independent PCR for hazelnut detection. A negative result in this independent assay would indicate that the positive amplification in the new or commercial PCR tests is due to cross-reactivity of the concerned species, leading to a false-positive result. If the sample would return a positive result in this assay it could be concluded that it was contaminated with hazelnut. The assay that was applied amplifies a non-coding mitochondrial sequence of 294 bp and has been developed for the detection of hazelnut in chocolate (Herman *et al.*, 2003). Only the pistachio sample returned a positive signal in this assay, although very faint (Figure 8, pistachio 1), indicating hazelnut contamination of these nuts. These results are also confirmed by melting curve analysis by performing this PCR reaction in real-time format with SYBR Green I detection.

Table VII Result of amplification of DNA of the selected food samples in new and commercial real-time PCR assays

| | PCR assay | Cor a 1 | Cor a 8 | First HN | Surefood HN | Gly m B 28 K | Gly M Bd 30 K | First Soy | Surfeod soy |
|---|---|---|---|---|---|---|---|---|---|
| | **Species** | | | | | | | | |
| **Protein BLAST** | Almond | - | - | - | - | - | - | - | - |
| | Barley | - | - | - | - | - | - | - | - |
| | Brazil nut | - | - | - | - | - | - | + | |
| | Cashew | - | - | - | - | - | - | - | - |
| | Coleseed | - | - | - | - | - | - | - | - |
| | Hazelnut | | | | | - | - | - | - |
| | Macademia | - | - | - | - | - | - | - | - |
| | Olive | - | - | - | - | - | - | - | - |
| | Pea | - | - | - | - | - | - | - | - |
| | Peanut | - | - | - | - | - | - | - | - |
| | Pecan | - | - | - | - | - | - | - | - |
| | Pistachio | - | - | + | - | - | - | - | - |
| | Wheat | - | - | - | - | - | - | - | - |
| **Prime r BLAST** | Lettuce | - | - | + | - | - | - | - | - |
| | Raspberry | - | - | + | + | - | - | - | - |
| | Spinache | - | - | - | - | - | - | - | - |
| | Strawberry | - | - | + | + | - | - | - | - |
| **Protein + primer BLAST** | Apple | - | - | - | - | - | - | - | - |
| | Grape | - | - | - | - | - | - | - | - |
| | Maize | - | - | - | - | - | - | - | - |
| | Oat | - | - | - | - | - | - | - | - |
| | Rice | - | - | - | - | - | - | - | - |
| | Soy | - | - | - | - | - | - | - | - |
| | Walnut | - | - | - | - | - | - | - | - |
| **Bulk ingredients** | Egg | - | - | - | - | - | - | - | - |
| | Kamut | - | - | - | - | + | + | + | + |
| | Milkpowder | - | - | - | - | - | - | - | - |
| | Spelt | - | - | - | - | - | - | - | - |
| | Starch | - | - | - | - | - | - | - | - |
| | Sugar | - | - | - | - | - | - | - | - |

The results from this independent PCR show that the positive signal with strawberry, raspberry and lettuce are the result of cross-reactivity of the primers of both commercial assays with these food/plant species. Interestingly, although these species were selected based on the results of the primer BLAST with the *Cor a 8* primers, no cross-reactivity was observed in this assay. Following, a new sample of pistachio nuts was purchased, indicating clearly on the package label that the product was free from other nuts. Nevertheless, the sample was first checked for

contamination with the independent hazelnut PCR, which returned a negative result (Figure 8, pistachio 2).

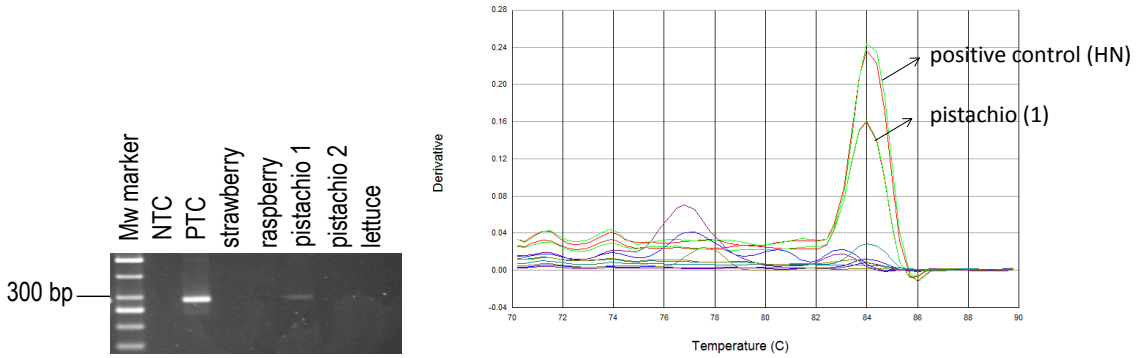


Figure 8 Independent hazelnut PCR - Left: conventional PCR with detection of PCR products by agarosegel electrophoresis and ethidium bromide staining. Right: melting curves obtained after amplification of DNA obtained from hazelnut (positive control), first pistachio sample (1), pistachio without contamination (2), raspberry, strawberry and lettuce.

Upon analysis of the sample in the First Hazelnut PCR a negative result was obtained, indicating that the positive result in the first analysis was due to the contamination. The small peaks that can be seen between 76 and 78 °C (Figure 8) originate from the reaction with DNA from the second pistachio sample. This signal could originate from the formation of primer dimers, which are typically seen at temperatures below the melting temperature of the amplicon. However, no primer dimers were observed in the negative control reaction, containing no DNA. The contamination of the first pistachio sample also explains the high signals measured with the hazelnut ELISA kits. Re-analysis of the second sample showed a tenfold reduction in the measured signal with the Veratox and Ridascreen FAST kit. The value obtained with the Hazelnut Residue kit was below the detection range. With the BioKits Hazelnut kit a value of 0.45 ppm was measured, which is above the LOD specified by the kit, indicating that this kit clearly suffers from cross-reactivity to pistachio. This cross-reactivity is however not specified by the kit's manual.

Both the new and commercial real-time PCR assays for soy showed amplification with the DNA obtained from the kamut sample (Table VII). The cross-reactivity that was observed in all three soy ELISA kits (Table V), already was an indication that this sample was contaminated with soy. To confirm this the *Gly m Bd 28K* and *Gly m* Bd 30K assays were repeated with SYBR Green I detection and melting curve analysis to reveal the identity of the amplified fragment. However, the results obtained by this approach were not unequivocal and could not be used to elucidate this question. This was done by sequencing the amplification products. Nucleotide BLAST of the PCR products obtained in the *Gly m* Bd 30K PCR returned the best similarity match with multiple database entries for the *Glycine max* Bd 30K gene. This result confirmed that the kamut sample was contaminated with soy. However, the Nucleotide BLAST of the *Gly m* Bd 28K PCR products showed highest similarity with a sequence from

*Triticum monococcum* (wild einkorn), an ancient wheat variety just like kamut itself. Similarity hits with the *Gly m* Bd 28K allergen gene were also found, but had less good scores. Based on this result one could conclude that the *Gly m* Bd 28K PCR shows cross-reactivity with wheat varieties. Analysis of the kamut sample in an independent soy PCR also confirmed the soy contamination. This assay is used in GMO detection and targets the lectin gene (Kuribara *et al.*, 2002). However, the cross-reactivity could not be confirmed with a newly purchased kamut sample. This sample was first verified for soy contamination in the lectin PCR and returned negative results upon analysis in as well both the *Gly m* Bd 28K and *Gly m* Bd 30K PCR as in the commercial soy PCR tests. A possible explanation for our findings could be that both kamut samples did not contain the same wheat variety, with the one variety showing cross-reactivity with the *Gly m* Bd 28K PCR and the other not. Kamut is actually a trademark for the wheat variety '*Triticum Korasan'*, which finds it origin in the ancient Egypt. The second sample was labelled to contain kamut, '*Triticum polonicum'*. The question is whether these two grains are the same or not. Analysis of the new, uncontaminated kamut sample in the commercial ELISA assays showed a residual cross-reactivity, only for the BioKits Soy assay (1.65 ppm).

Contamination of the Brazil nuts with soy was also demonstrated with the independent PCR test. No sequence analysis of the PCR products could be performed due to the lack of the primer sequences used in the commercial assays. Analysis of the newly purchased Brazil nut samples showed no cross-reactivity in all real-time PCR assays and also in the ELISA kits.

### 2.2.3 Impact of food processing simulating reactions on the detectability by commercial ELISA

During food processing, modifications on allergen level might occur which can alter the antigen antibody interactions in the routinely used ELISA therefore leading to erroneous results. A careful screening of the performance of the commercial ELISA with hazelnut and soybean proteins modified through food processing simulating reactions was performed. The Maillard reaction in the presence or absence of other proteins, protein oxidation in the presence or absence of lipids and partial protein hydrolysis was therefore induced in buffered systems. These reactions were induced in buffered systems in order to make abstraction of the protein extractability which might have an additional effect on the obtained results.

Proteins were extracted from the hazelnuts and soybeans with the selected protein isolation protocol (see 2.1.1). Protein content was determined according to the Kjeldahl procedure (AOAC Official Method 981.10, 1981). A factor of 5.41 was used to convert nitrogen to hazelnut protein and 5.71 to convert to soybean proteins. The non protein nitrogen (NPN) was determined in the supernatant after a previous protein precipitation with 15 % TCA (final concentration).

**Maillard reaction**

One of the most important protein modifications induced by thermal processing and long storage is the Maillard reaction, which involves reaction between amino acids and reducing sugars (Contreras-Calderon *et al.*, 2009). There is abundant evidence that thermal processing and non-enzymatic browning reactions can have a great impact on the IgE-binding activity of food allergens. However, the effects observed are highly dependent on the type of allergen studied (Gruber *et al.*, 2005; Gruber *et al.*, 2004; Nakamura *et al.*, 2005; Nakamura *et al.*, 2008). In contrast, limited information regarding the impact of the Maillard reaction on the detectability by commercial ELISA is available.

The model systems without wheat proteins were prepared in 0.1 M phosphate buffer pH 7.4 by mixing 1 % (w/v) hazelnut or soybean proteins with 6 % (w/v) glucose. The mixtures were loaded in closed falcon tubes that were incubated in a water bath at 70°C for up to 48 h. The model systems with wheat proteins were prepared in the same buffer. Two types of model systems were considered: (1) 0.25 % (w/v) of hazelnut/soybean proteins with 0.75 % (w/v) of wheat proteins with our without 6 % (w/v) of glucose, (2) 0.5 % (w/v) hazelnut/soybean proteins with 0.5 % (w/v) wheat proteins with or without 6 % (w/v) glucose.

The impact of the Maillard reaction was evaluated first on protein level. Protein bound carbonyls were measured after converting them to the corresponding 2, 4-dinitrophenylhydrazones (Cucu *et al.*, 2010b). The carbonyl content was calculated using a molar absorption coefficient of 22000 $M^{-1}$ $cm^{-1}$ (Levine *et al.*, 1994). The loss of reactive lysine residues was monitored using derivatization with *ortho*-phthaldialdehyde (OPA) in the presence of 2–mercaptoethanol as previously described *(Morales et al., 1996).* Free amino groups were determined using derivatization with 2, 4, 6-trinitrobenzene sulfonic acid (TNBS) as previously described (Fields, 1972). The oxidation of the sulfur containing amino acids was monitored by their derivatization with 5.5'-dithiobis-2-nitrobenzoate (DTNB) as previously described (Beveridge *et al.*, 1974). Total thiol groups were determined in a similar manner after a previous reduction of the disulfide bridges with β–mercaptoethanol.

The Maillard reaction either in the presence or absence of other proteins (soluble wheat proteins) led to severe changes on hazelnut and soybean protein level. The interaction of reducing sugars or dicarbonyl compounds derived from sugars led to the formation of protein-bound carbonyls with a simultaneous decrease of available lysine residues (Figure 9 & Figure 10).

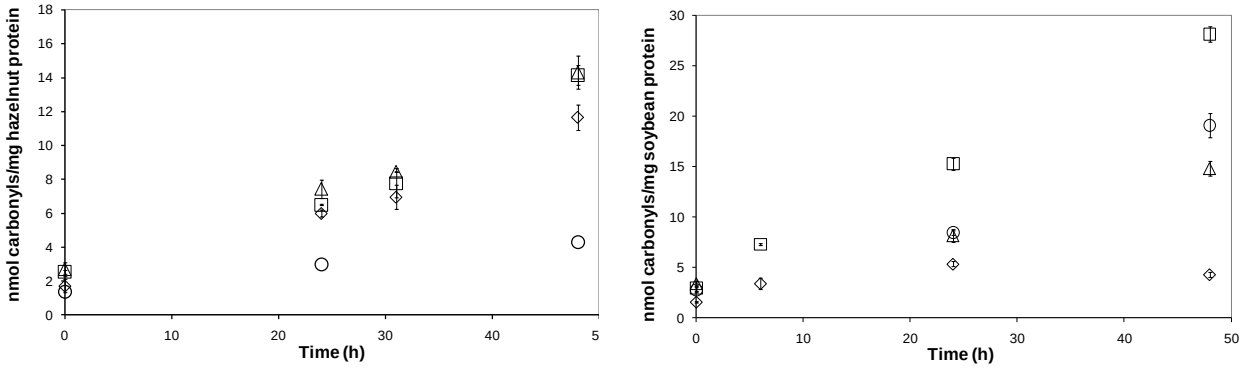


Figure 9. Formation of protein bound carbonyls in soybean protein in the presence or absence of soluble wheat proteins due to incubation with glucose. The data points represent mean values ±SD of three independent determinations. □ - 1% hazelnut or soybean proteins with 6% glucose; ○ - 0.5% hazelnut or soybean with 0.5% soluble wheat proteins and 6% glucose; Δ 0.25% hazelnut or soybean and 0.75% soluble wheat proteins and 6% glucose; ◊ 1% hazelnut or soybean proteins

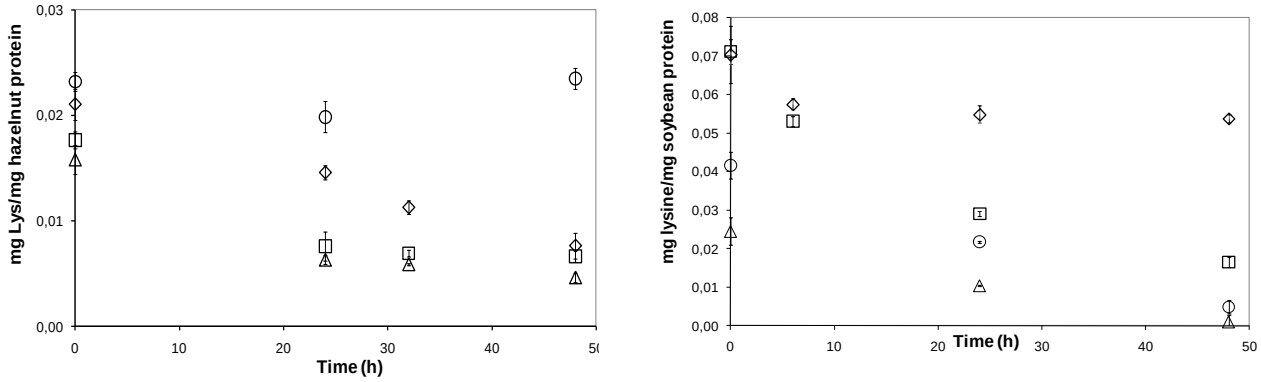


Figure 10 Formation of protein bound carbonyls in soybean protein in the presence or absence of soluble wheat proteins due to incubation with glucose. The data points represent mean values ±SD of three independent determinations. □ - 1% hazelnut or soybean proteins with 6% glucose; ○ - 0.5% hazelnut or soybean with 0.5% soluble wheat proteins and 6% glucose; Δ 0.25% hazelnut or soybean and 0.75% soluble wheat proteins and 6% glucose; ◊ 1% hazelnut or soybean proteins

Moreover changes in the electrophoretic pattern of the hazelnut and soybean proteins as result of incubation with glucose were observed. Most of the allergenic proteins have been affected as manifested by the loss of intensity of their respective bands. The intensity of the bands representing hazelnut and soybean proteins decreased with the incubation time and smearing towards the top of the gel progressed. Incubation for 48 h led to aggregates formation with even much bigger masses: clearly, their migration into the running gel was hindered. Since SDS-PAGE experiments were performed under reducing conditions, the observed protein aggregation cannot be due to disulfide formation. This was most probably due to the covalent bounds formed between the formed carbonyls and the available α- or ε - amino groups. Nevertheless, some allergenic proteins turned to be fairly stable under the investigated conditions. The acid and basic subunits of the soybean derived *Gly*

*m 6* allergen (32 and 19 kDa, respectively) remained stable upon the whole incubation time in the absence of glucose. During incubation with glucose, the intensity of the bands representing *Gly m 6* is decreasing with the incubation time. The hazelnut derived *Cor a 9* allergen (a 11S globulin with acidic (35-40 kDa) and basic (~20kDa) subunits) was stable during Maillard reaction either in the presence or absence of soluble wheat proteins (Figure 11).

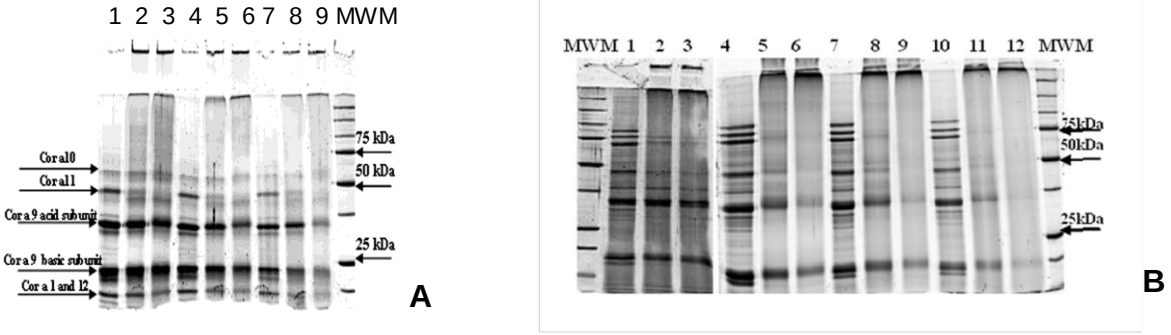


Figure 11 SDS-PAGE pattern of the hazelnut (A) and soybean proteins (B) incubated in the presence or absence of wheat proteins with glucose. A Lane 1 -3 1% hazelnut proteins and 6% glucose non treated, 24 h and 48 h incubated resp., line 4 – 6 0,5% hazelnut proteins with 0,5% wheat proteins and 6% -glucose non treated, 24 h and 48 h incubated resp., lane 7-9 0,25% hazelnut proteins with 0,75% wheat proteins and 6% glucose non treated, 24 h and 48 h incubated resp., B Lanes 1-3: 1% soybean proteins non-treated, 24 h and 48h incubated resp.; lanes 4-6: 1 % hazelnut or soybean proteins and 6 % glucose non-treated, 24 h and 48 h incubated resp.; lanes 7-9: 0.5 % soybean proteins with 0.5 % wheat proteins and 6 % glucose non-treated, 24 h and 48 h incubated resp.; lanes 10-12: 0.25 % soybean proteins with 0.75 % wheat proteins and 6 % glucose non-treated, 24 h and 48 h incubated resp.; MWM - molecular weight marker.

Further, the influence of the protein modifications induced by the Maillard reaction on their detection was investigated. To this end, samples of the different model systems were analyzed in the different commercial ELISA kits (Table II). For each sample of the different model systems a dilution series was made in the extraction buffer/dilution solution of the respective kit. The actual concentrations are the concentrations of hazelnut/soybean proteins based on the absolute protein determination using the Kjeldahl procedure and do not include the wheat proteins that were added in some of the model systems. The dilution series of the samples were analysed in duplicate in the different kits according to the manufacturers' instructions. The concentration of the standards included in three of the four kits tested for hazelnut detection (Veratox® for hazelnut, Ridascreen® FAST Hazelnut and BioKits Hazelnut Assay) is expressed as a particular amount of *hazelnut*. However, the samples of the different prepared model systems have a known or actual *hazelnut protein* concentration. To be able to compare the actual concentration of the samples that were analysed with the measured concentration derived from the calibration curve obtained with the kit's standards, the concentration

of the standards was converted to the same dimension of that from the model system samples. This was done according to the formula below (2), taking into consideration the protein content of the hazelnut standards as indicated in the manual of the kit or, if not indicated, a protein content of 12% was used (Belser *et al.*, 1999).

The content of the standards of the kits used for soybean detection was already expressed as a protein concentration, so this conversion was not required here.

$$concentration\ hazelnut \times protein\ content\ \ \% \ = concentration\ hazelnut\ protein \quad (2)$$

The hazelnut or hazelnut/soybean protein concentration of the kit's standards is expressed in parts per million (ppm) which equals ng analyte/mg food product. However, as the concentration of the hazelnut/soybean proteins in the model systems is expressed in ng/mL, the concentrations of the kit's standards were converted according to formula 3, considering the weight of the sample (mg) extracted by a particular volume of extraction buffer (mL) according to the respective manufacturers' instructions.

$$\frac{ng\ analyte}{mg\ food}\ \ ppm\ \ \times \frac{weight\ of\ extracted\ sample\ \ mg}{volume\ of\ extraction\ buffer\ used\ \ ml} = \frac{ng\ analyte}{ml\ solution} \quad (3)$$

Data above or below the calibration range are indicated by an asterisk (*) or a 'zero' (0), respectively, in the charts.

Analysis of the reference hazelnut protein extract in the various kits yielded remarkable differences in performance. The Ridascreen Fast Hazelnut kit proved to be the most accurate, while the Veratox for Hazelnut kit showed results that underestimated by one order of magnitude the actual hazelnut protein present in the reference extract. Application of the reference soybean protein solution in the various kits resulted in an extreme overestimation of the actual protein concentration in the Veratox Soy Allergen and BioKits Soya assays, while for the Soy Residue assay an underestimation was recorded. A possible explanation for the differences between the actual and the measured protein concentrations that were obtained with the different kits could be found in the nature of the standard solutions and antibodies of the respective kits.

Heating the reference hazelnut protein solution in the absence of glucose, which restricted the Maillard reaction as confirmed from the Maillard reaction parameters monitored, further decreased the accuracy for the Veratox for Hazelnut kit and the Biokits Hazelnut assay. In the case of the Hazelnut Residue kit a slightly increased accuracy was seen. Upon heating the reference soybean protein solution in absence of glucose, a decreased detection was observed in the BioKits Soya

Allergen, however, still overestimating the actual soybean protein concentration. In contrast, both the Veratox Soy Allergen and the Soy Residue assay lacked total robustness. The complete loss of detectability can probably be due to the denaturation of soybean proteins especially because only minor changes on protein level were observed compared to the changes observed in the model systems containing glucose (Figure 10 and Figure 11).

When hazelnut or soybean proteins are present in a food product, protein cross-linking can occur with other (bulk) proteins, which could have an additional impact on the detection. Therefore the impact of heating hazelnut or soybean proteins in the presence of soluble wheat proteins was investigated as well. While an additional impact could be seen for the hazelnut kits, addition of the soluble wheat proteins had no effect on the detection of the soybean proteins.

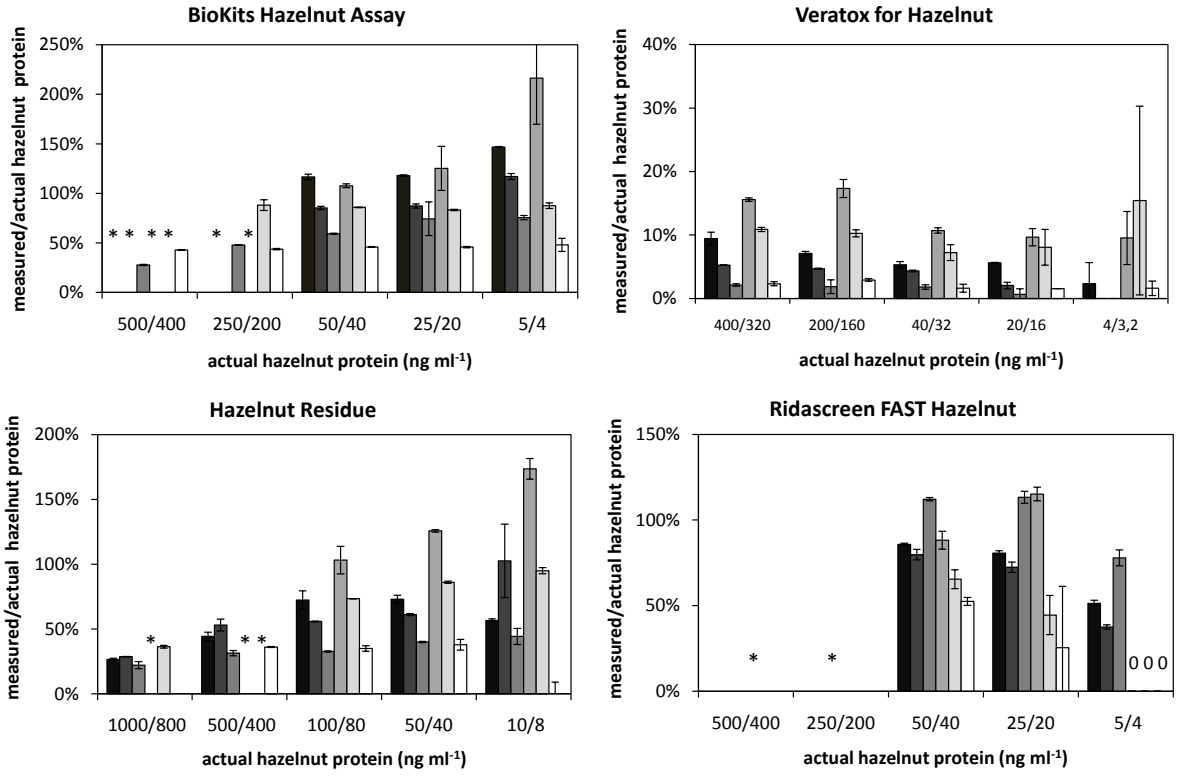


Figure 12 Ratio of measured over actual hazelnut protein concentration (Y-axis) in different commercial ELISA kits after duplicate analysis of a dilution series (X-axis) of a reference hazelnut protein sample (darkest) and after heating in the presence or absence of glucose and in the absence (left concentration on X-axis) or presence (right concentration on X-axis) of soluble wheat proteins. (* = absorbance value of sample outside the range of the standard curve) ■ reference, ■ reference, heated 48 h, ■ reference+glucose, heated 48 h, □ 0.25% hazelnut proteins+0.75% soluble wheat proteins, □ 0.25% hazelnut proteins+0.75% soluble wheat proteins heated 48 h, □ 0.25% hazelnut proteins+0.75% soluble wheat proteins+glucose, heated 48 h.

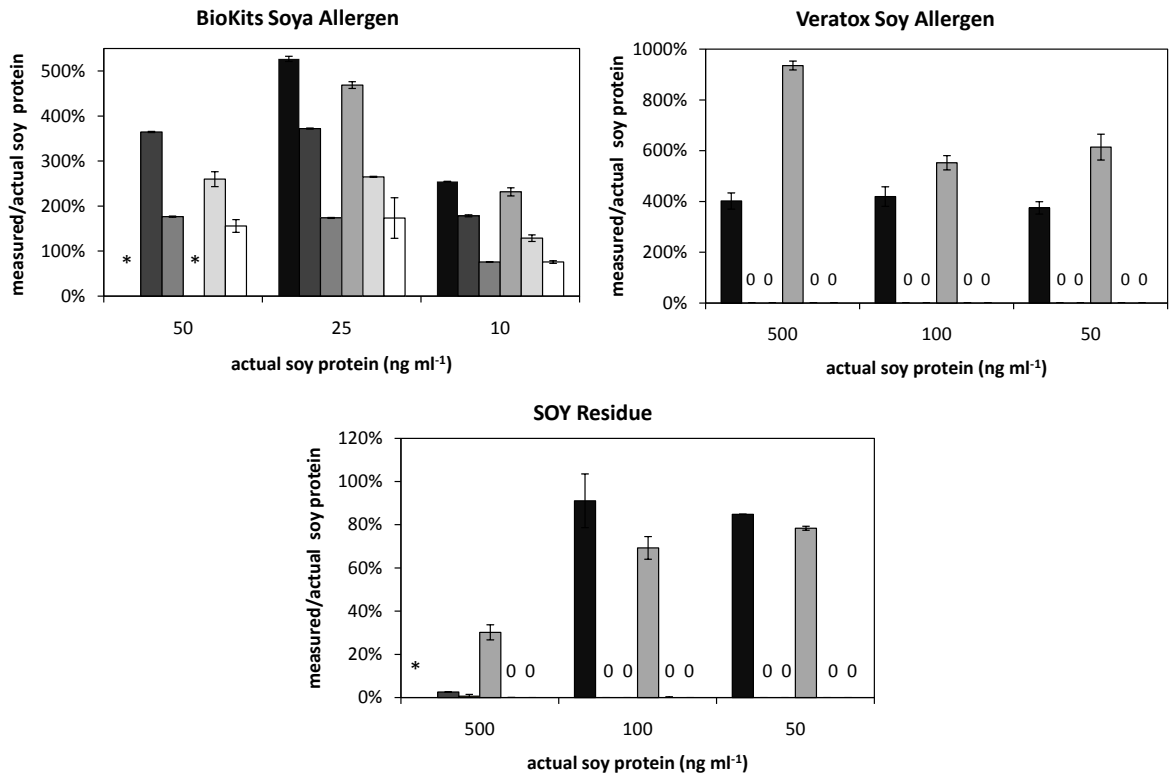


Figure 13 Ratio of measured over actual soybean protein concentration in different commercial ELISA kits after duplicate analysis of a dilution series of a reference soybean protein solution and after heating in the presence or absence of glucose and soluble wheat proteins. (* = absorbance value of sample above the calibration range, 0 = absorbance value of sample below the calibration range). ■ -reference sample, ■ -reference heated for 48 h, ■ -reference heated for 48 h in the presence of glucose, ■ -0.25 % soybean proteins and 0.75 % soluble wheat proteins, □ -0.25 % soybean proteins and 0.75 % soluble wheat proteins heated for 48 h, □ -0.25 % soybean proteins and 0.75 % soluble wheat proteins heated for 48 h in the presence of glucose

The changes on the protein level that took place upon heating the proteins in the presence on glucose led to further decreases in detectability for all kits, except the Ridascreen FAST Hazelnut. With this kit even a better binding occurred between the antibodies and the Maillard modified hazelnut proteins compared to the proteins in the non-heated reference extract, most probably indicating that the antibodies used in that assay alone were developed for modified hazelnut proteins. Nevertheless, in the same kit, additional protein cross-linking with other soluble wheat proteins during glycation, did seem to affect the detectability of the hazelnut proteins. Similar impact of the cross-linking with the wheat proteins could be seen for the BioKits Hazelnut Assay, while it did not seem to affect the hazelnut protein detection with the Veratox for Hazelnut and the BioKits Hazelnut Assay. As a complete lack of robustness of the Veratox Soy Allergen and the Soy Residue was observed upon heating soybean proteins alone, no additional impact of their heating with glucose either in the presence or absence of soluble wheat proteins could be seen.

**Protein oxidation**

Little information is now available regarding the impact of protein oxidation on their detectability. Nevertheless, it was shown that the incubation of soybean globulins with oxidized oil led to an increase in the allergenicity (Doke *et al.*, 1989).

A comparison between the oxidation in the presence of lipids and in the presence of hypochlorous acid was made because each oxidation process induces changes on protein level via different routes and their impact on the detectability was assessed. The interaction of lipids and proteins may affect various properties of food products and is favored 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. These compounds are responsible for unacceptable flavors and cause many changes in the proteins as a result of their interaction with with lysine's amino groups, cysteine's thiol groups and histidine's imidazole groups therefore leading to an increase in protein bound carbonyls (Refsgaard *et al.*, 2000). Several amino acids, mainly cysteine, methionine, histidine, tryptophan, tyrosine, and lysine, were shown to be affected by the oxidation products of lipids, resulting in a reduced bioavailability. It is also possible that direct oxidation of the proteins via the hydroxyl radicals formed during lipid oxidation (by thermal dissociation of the hydroperoxides) might lead to the formation of protein radicals by abstraction of hydrogen and therefore leading to the formation of cross linked products. On the other hand, protein radicals, can also react with oxygen and produce protein peroxy radicals and then protein hydroperoxides by abstracting hydrogen from other molecules. Protein hydroperoxides can then be decomposed in the presence of transition metals and by cleavage via the diamide or α-amidation pathways, protein carbonyls can be formed (Berlett and Stadtman, 1997; Choe and Min, 2006).

Hypochlorous acid was used because it reacts rapidly with amino acids and protein oxidation can be achieved in well controlled conditions, therefore allowing to investigate its impact on proteins. Hypochlorous acid can readily attack proteins and form unstable chloramines on the α-amino group and the side chain of the lysine residues which can further decompose with the formation of protein carbonyls. Moreover, amino acids such as cysteine, methionine, histidine, tryptophan and tyrosine can be modified by the interaction with hypochlorous acid with the formation of such products as disulfide bridges, methionine sulfoxide, kynurenine and dityrosine (Hawkins *et al.*, 2003).

Lipid induced protein oxidation was achieved by means of incubation of proteins with oils with different initial oxidation status and oils with different unsaturation degrees. Fresh soybean oil had a p-AV value of 4.16 while the slightly and highly oxidized soybean oils had a p-AV value of 10.33 and 93.89 respectively. To evaluate the impact of incubation of soybean proteins with oils with different unsaturation degrees, fresh olive, soybean, sunflower and fish oil with a were used.

To characterize the oils, determination of the fatty acid composition was done by means of gas chromatography (AOCS Official Method Ce 1b-89, 1989). Oxidation status of the oils used in the reaction systems was followed by iodometric determination of the peroxide value (AOCS Official Method Cd 8b-90, 1989). Soybean oil was oxidized under UV light (235 nm) at 35 °C for up to 48 h. The amount of secondary oxidation products in the oils was determined by measuring their p-anisidine values (p-AV) (AOCS Official Method Cd 18-90, 1989).

The lipid induced reaction systems were prepared in 50 mM 3-morpholinopropanesulfonic acid (MOPS) pH 7.4 by mixing 1% (w/v) oil with 2% (w/v) hazelnut or soybean protein and incubated at 70°C in the presence of 10 µM copper sulfate solution to initiate the oxidation. 0.2 g/L sodium azide was added to prevent microbial growth. After incubation, samples were defatted 3 times with technical hexane. The defatted proteins were precipitated with 15% TCA (final concentration), the pellet was redissolved in 2% SDS and the pH was adjusted to approximately 10 with 10 M NaOH to facilitate the solubilisation. Undissolved particles were removed by centrifugation at 10000 g for 10 min. The clear supernatant was used for analysis.
For the direct oxidation, 200 µl protein solution (10 mg/mL) in 100 mM phosphate buffer pH 8 or 5.8 was oxidized with 50 µL hypochlorous acid (0 - 5 mmol/g protein) at 30°C for 10 min. The initial concentration of the hypochlorous acid was determined by iodometric titration. After treatment, the samples were immediately immersed in ice.

Protein bound carbonyls, available lysine and total thiol groups were assessed as previously described. Amino acid analysis of the oxidized protein samples was carried out after acid or basic hydrolysis. A treatment with 34 mM $NaBH_4$ for 4 h at 37 °C was performed in order to stabilize the protein – lipid adducts and to make them resistant to acid hydrolysis (Sanchez-Vioque *et al.*, 1999). For the hypochlorous acid induced oxidation, no pre-treatment with $NaBH_4$ was performed. Further, the samples were hydrolyzed with 6 M HCl for 24 h at 110 °C. For tryptophan determination alkaline hydrolysis was performed with 4.2 M NaOH for 24 h at 110 °C. Hydrolysates were neutralized with NaOH or HCl to bring the pH to 2.2 and 4.25 respectively. The HPLC chromatographic system employed consisted of an Agilent 1100 model (Agilent Technologies, Diegem, Belgium). The chromatographic column was ZORBAX Eclipse AAA Rapid Resolution column 4.6 x 150 mm, 3.5 micron (Agilent Technologies, Diegem, Belgium) operated at 40 °C at a flow rate of 2 mL $min^{-1}$. The chromatographic separation was achieved by injecting 0.5 µl sample and using a

gradient elution of mobile phase A (40 mM $NaH_2PO_4$ with 0.2 g $L^{-1}$ $NaN_3$ pH 7.8) and mobile phase B (acetonitrile/methanol/water in a 45/45/10 ratio) and allowed separation of the amino acids in 28 min (Agilent application note). Primary amino acids were derivatized with 0.5 µL of 75 mM OPA and detected at excitation and emission wavelengths 340/450 nm. The secondary amino acid proline was derivatized with 0.5 µL of 10 mM 9-fluorenyl-methyl-chloroformate (FMOC) and detected at excitation and emission wavelength of 266/305 nm. Internal standards norvaline and sarcosine were used for the quantification.

The major PUFA's in the fish oil were docosahexaenoic acid (DHA; 22:6 ω-3) and eicosapentaenoic acid (EPA; 20:5 ω–3). On the other hand, the soybean and sunflower oil contained mainly linoleic acid (18:2 n–6) with soybean oil containing also some linolenic acid (18:3 ω-3). The olive oil contained about 73% of monounsaturated oleic acid (18:1). Based on the fatty acids profile, the theoretical amount of double bonds (mmol in 100 g fatty acids) represented 332.5, 528.1, 522.6 and 708.7 for olive, soybean, sunflower and fish oil respectively.

Although it is believed that controlled oxidation may improve physico-chemical properties of some proteins (Liu and Xiong, 2000), oxidation obviously has a negative impact on proteins as well. Protein oxidation, either lipid or hypochlorous acid induced, led to severe changes on protein level as evidenced by formation of protein bound carbonyls. The ability of oils to promote carbonyl formation increases in the order fresh olive, soybean < soybean p-AV 10.33 < sunflower < fish oil and soybean p-AV 93.89. However, the amount of protein bound carbonyls formed as a results of hypochlorous acid induced oxidation was about 5 times higher as compared to lipid induced oxidation. Additionally, severe changes in the electrophoretic pattern of the proteins were observed (Figure 14-16). Nevertheless, same proteins (*Cor a* 9 and *Gly m* 6) were the most stable under the oxidizing conditions. These proteins turned to be completely modified only upon severe hypochlorous acid induced oxidation (3.755 to 5 mmol hypochlorous acid/g protein).

During hypochlorous acid induced oxidation, severe losses of histidine, arginine, tyrosine methionine and tryptophan residues were observed (Table VIII & Table IX). Lipid induced oxidation led to less pronounced modifications on amino acid level with lysine and histidine residues being the most affected probably via the adduction with secondary oxidation products. Modification of methionine, tryptophan and tyrosine residues took place only under the most severe oxidizing conditions (incubation with highly oxidized soybean and fresh fish oil) and were probably directly oxidized by hydroxyl radicals formed during thermal dissociation of lipid hydroperoxides or lipid hydroperoxides-derived alkoxy- and peroxy radicals.

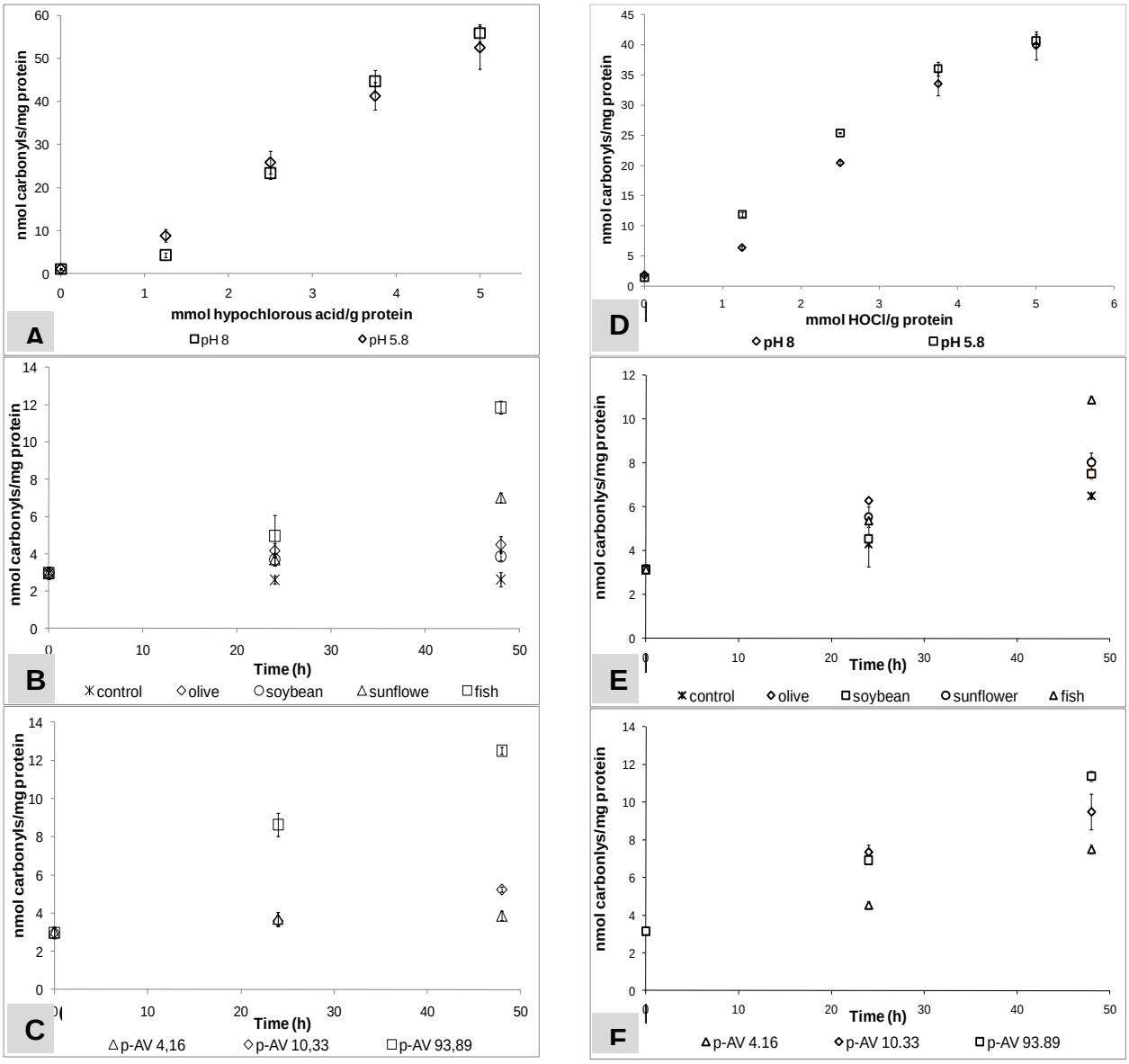


Figure 14 Protein carbonyl formation upon hypochlorous acid induced oxidation (A – soybean and D – hazelnut proteins) and lipid induced oxidation using oils with different unsaturation degree (B – soybean and E – hazelnut proteins) and different initial oxidation status (C – soybean and F - hazelnut proteins); data points represent mean of 3 independent determinations ± SD

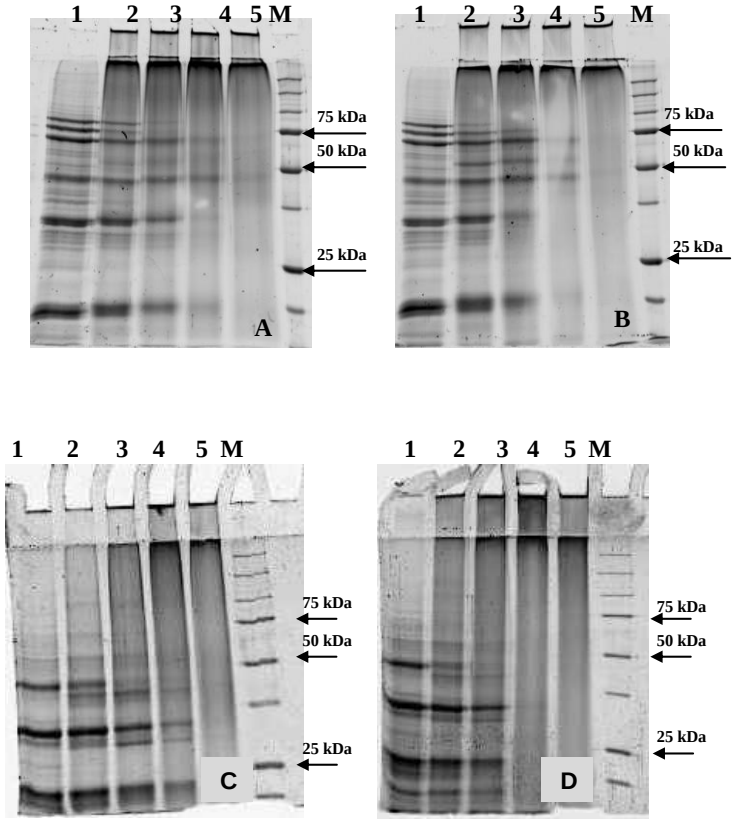


Figure 15 Soybean (A & B) and hazelnut proteins (C & D) oxidized with hypochlorous acid at pH 8 and 5.8 respectively. Lane 1 – native soybean or hazelnut proteins; lanes 2 – 5: oxidized with 1.25, 2.5, 3.75 and 5 mmol hypochlorous acid/g protein; M- molecular weight marker.

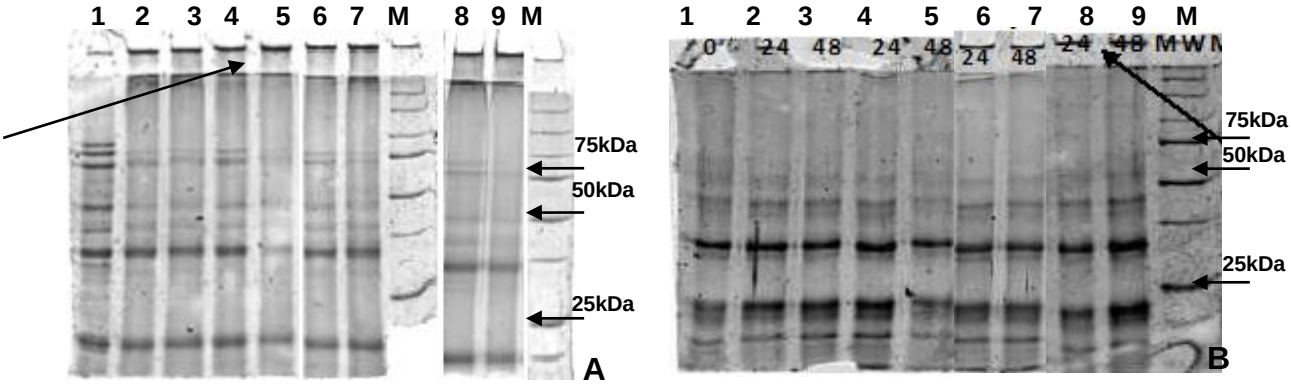


Figure 16 Soybean (A ) and hazelnut proteins (B) oxidized with oils: lane 1 – control, lane 2 & 3 – olive oil for 24 and 48 h; lane 4 & 5 – fish oil for 24 and 48 h; lane 6 & 7– soybean oil p-AV 93.89 for 24 and 48 h; lane 8 & 9 – no oil for 24 and 48 h. M - molecular weight marker. Arrow indicating high molecular weight aggregates

Table VIII Effect of hypochlorous acid, oxidized soybean oil (p-AV 93.89) and fresh fish oil induced oxidation on the amino acid composition of soybean protein (g/100 g protein). Data points represent mean values of 3 independent determinations. Values in the same line followed by different letters are significantly different (p<0.05)

| Control | | mmol hypochlorous acid/g protein | | | | | | | | Soybean oil | Fish oil |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0.625 | 1.25 | 2.5 | 5 | 0.625 | 1.25 | 2.5 | 5 | | |
| | | pH 8 | | | | pH 5.8 | | | | | |
| **His** | 2.53 $^{a}$ | 2.74 $^{b}$ | 2.73 $^{b}$ | 2.24 $^{c}$ | 1.21 $^{e}$ | 2.72 $^{b}$ | 2.64 $^{b}$ | 2.06 $^{d}$ | 0.39 $^{f}$ | 1.95 $^{d}$ | 1.90 $^{d}$ |
| **Arg** | 7.57 $^{a}$ | 8.00 $^{b}$ | 7.96 $^{b}$ | 7.13 $^{a}$ | 4.56 $^{d}$ | 7.99 $^{b}$ | 8.11 $^{b}$ | 7.88 $^{b}$ | 5.90 $^{c}$ | 6.22 $^{c}$ | 6.05 $^{c}$ |
| **Tyr** | 3.75 $^{a}$ | 3.94 $^{b}$ | 3.81 $^{ab}$ | 2.65 $^{d}$ | 0.59 $^{e}$ | 3.90 $^{b}$ | 3.74 $^{ab}$ | 2.60 $^{d}$ | 0.38 $^{e}$ | 3.19 $^{c}$ | 3.05 $^{c}$ |
| **Met** | 1.49 $^{ab}$ | 1.52 $^{a}$ | 1.46 $^{abc}$ | 1.25 $^{e}$ | 0.29 $^{g}$ | 1.37 $^{cd}$ | 1.04 $^{f}$ | 0.00 $^{h}$ | 0.00 $^{h}$ | 1.40 $^{bcd}$ | 1.36 $^{d}$ |
| **Lys** | 6.28 $^{a}$ | 6.33 $^{a}$ | 6.53 $^{a}$ | 5.42 $^{b}$ | 5.01 $^{bc}$ | 6.48 $^{a}$ | 6.39 $^{a}$ | 5.47 $^{b}$ | 5.46 $^{b}$ | 4.76 $^{c}$ | 4.46 $^{c}$ |
| **Trp** | 0.96 $^{a}$ | 0.89 $^{ab}$ | 0.65 $^{cd}$ | 0.35 $^{e}$ | 0.06 $^{f}$ | 0.77 $^{bc}$ | 0.61 $^{d}$ | 0.22 $^{e}$ | 0.00 $^{f}$ | 0.69 $^{cd}$ | 0.67 $^{cd}$ |

Table IX Effect of hypochlorous acid, oxidized soybean oil (p-AV 93.89) and fresh fish oil induced oxidation on the amino acid composition of hazelnut protein (g/100 g protein). Data points represent mean values of 3 independent determinations. Values in the same line followed by different letters are significantly different (p<0.05)

| Control | | mmol hypochlorous acid/g protein | | | | | | Soybean oil | Fish oil |
|---|---|---|---|---|---|---|---|---|---|
| | | 1.25 | 2.5 | 5 | 1.25 | 2.5 | 5 | | |
| | | pH 8 | | | pH 5.8 | | | | |
| **His** | 2.31 $^{a}$ | 2.13 $^{b}$ | 1.95 $^{c}$ | 1.11 $^{h}$ | 1.51 $^{f}$ | 1.28 $^{g}$ | 0.30 $^{i}$ | 1.65 $^{e}$ | 1.78 $^{d}$ |
| **Arg** | 15.93 $^{a}$ | 15.13 $^{ab}$ | 14.32 $^{b}$ | 7.61 $^{e}$ | 14.55 $^{b}$ | 15.08 $^{b}$ | 8.99 $^{d}$ | 12.48 $^{c}$ | 13.05 $^{c}$ |
| **Tyr** | 3.36 $^{a}$ | 2.90 $^{b}$ | 1.32 $^{d}$ | 0.25 $^{f}$ | 1.56 $^{d}$ | 0.96 $^{e}$ | 0.13 $^{f}$ | 2.50 $^{c}$ | 2.63 $^{c}$ |
| **Met** | 1.67 $^{a}$ | 1.27 $^{b}$ | 0.47 $^{cd}$ | 0.52 $^{c}$ | 0.34 $^{cd}$ | 0.25 $^{d}$ | 0.00 $^{e}$ | 1.29 $^{b}$ | 1.33 $^{b}$ |
| **Lys** | 2.53 $^{ab}$ | 2.61 $^{a}$ | 2.50 $^{abc}$ | 2.41 $^{bcd}$ | 2.32 $^{d}$ | 2.33 $^{d}$ | 2.39 $^{cd}$ | 1.61 $^{e}$ | 1.73 $^{e}$ |
| **Trp** | 1.00 $^{a}$ | 0.36 $^{c}$ | 0.43 $^{c}$ | 0.10 $^{e}$ | 0.41 $^{c}$ | 0.22 $^{d}$ | 0.00 $^{f}$ | 0.81 $^{b}$ | 0.96 $^{a}$ |

Figure 17 shows the results of the analysis of samples of the HOCl-induced protein oxidation in the hazelnut ELISA kits. Analysis of the untreated reference hazelnut protein sample in the different kits showed that the actual hazelnut protein concentration was underestimated with the Veratox for Hazelnut and the Ridascreen FAST hazelnut kits. In contrast, the other two kits overestimated the actual hazelnut protein concentration in the reference sample. As soon as oxidation was induced almost no or no hazelnut proteins were detected anymore, regardless the pH at which oxidation was induced. Interestingly, with the Ridascreen FAST Hazelnut kit the proteins were better detected upon oxidation at pH 5.8 than at pH 8.

The actual soybean protein concentration in the reference sample was seriously overestimated with the Veratox Soy Allergen and BioKits Soy Allergen kits (Figure 18). With the Soy Residue kit the detection of the native soybean proteins was concentration-dependent, leading towards a stronger underestimation as the concentration of soybean proteins in the sample decreased. In general, a slightly lower amount of soybean proteins was detected at pH 5.8 compared to the detection at pH 8 with all kits. Similarly as for the hazelnut proteins, oxidation with

hypochlorous acid strongly reduced the detection of the soybean proteins, however, no clear influence of the pH could be seen.

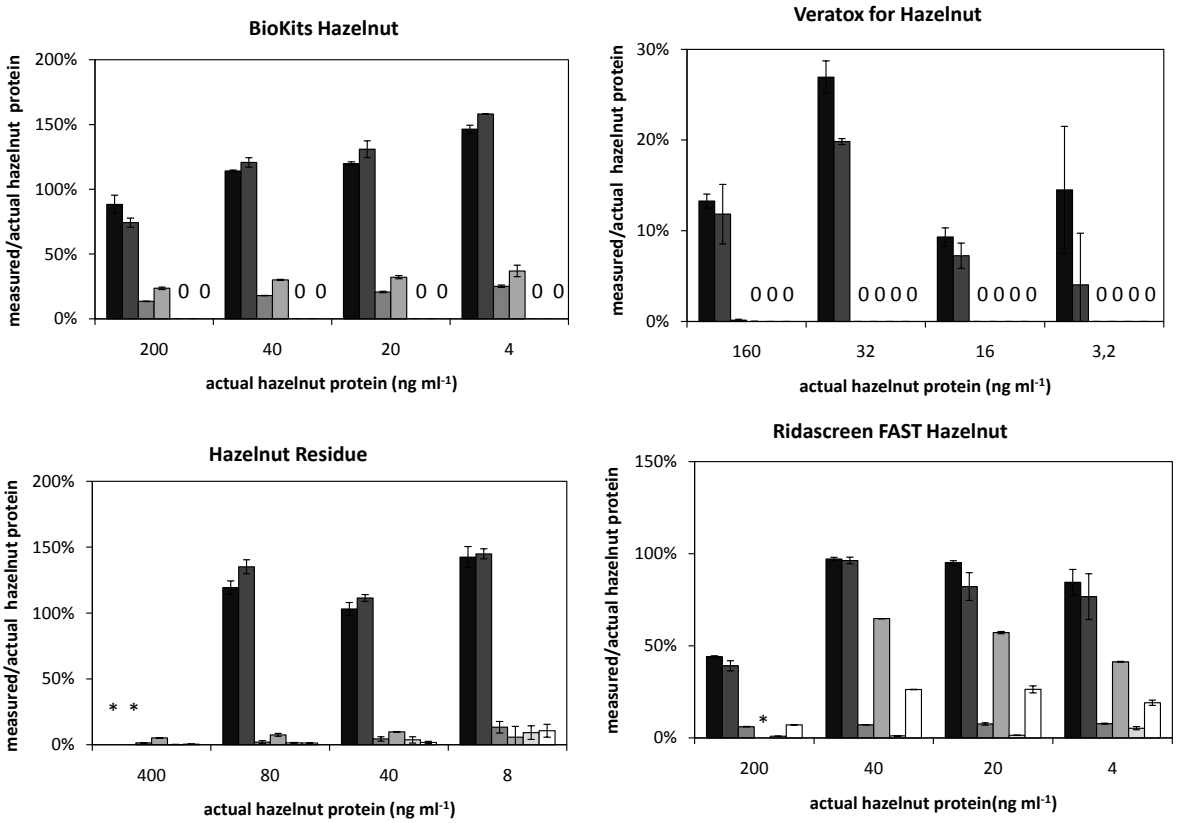


Figure 17 Ratio of measured over actual hazelnut protein concentration (y-axis) in different ELISA kits after duplicate analysis of a dilution series (x-axis) of an untreated hazelnut protein sample and after oxidation with hypochlorous acid at pH 8 and 5.8. (* = absorbance value of sample above the calibration range, 0 = absorbance value of sample below the calibration range). ■ - 0 mM/g protein, pH 8, ■ - 0 mM/g protein, pH 5.8, ■ - 2.5 mM/g protein, pH 8, ■ - 2.5 mM/g protein, pH 5.8, □ - 5mM/g protein pH 8, □ - 5 mM/g protein, pH 5.8.

Figure 19 shows the results of the analysis of samples of lipid-induced protein oxidation in the hazelnut ELISA kits. The actual hazelnut protein concentration in the untreated reference samples (with or without lipids) was underestimated by all hazelnut ELISA kits. From this figure it can be seen that the presence of the lipids does not interfere with the detection. With the BioKits Hazelnut assay, the reference and oxidised samples returned absorbance values that were below the calibration range. Consequently, no conclusion could be made with regard to the influence of protein hydrolysis on the detection of hazelnut proteins with this kit. Possibly some step in the preparation of the samples was interfering with the antibody binding.

We can conclude that the altered detection upon incubation at 70°C (overall decreased detection after 48 h incubation at 70°C) can mainly be attributed to protein denaturation as no clear influence of the lipid induced oxidation could be observed.

Similarly as described above for the reference samples of the HOCl-induced oxidation, the Veratox and BioKits Soy Allergen assays highly overestimated the actual soybean proteins (Figure 20). With the Soy Residue kit these samples were underestimated. While heat treatment (almost) completely destroyed the detection of the soybean proteins with the Veratox Soy Allergen and Soy Residue kit, an increased detection was registered for the BioKits Soy Allergen kit, regardless the presence of lipids.

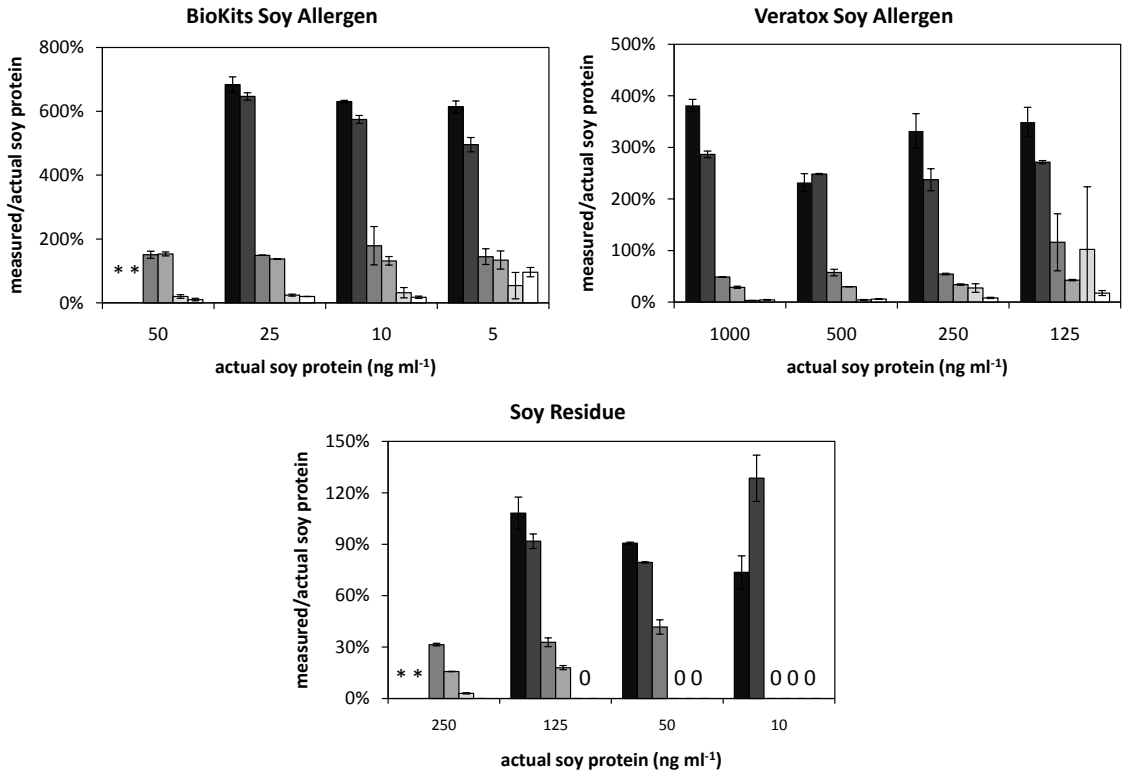


Figure 18 Ratio of measured over actual soybean protein concentration (y-axis) in different ELISA kits after duplicate analysis of a dilution series (x-axis) of an untreated soybean protein sample and after oxidation with hypochlorous acid at pH 8 and 5.8. (* = absorbance value of sample above the calibration range, 0 = absorbance value of sample below the calibration range). ■ - 0 mM/g protein, pH 8, ■ - 0 mM/g protein, pH 5.8, ■ - 2.5 mM/g protein, pH 8, ■ - 2.5 mM/g protein, pH 5.8, □ - 5mM/g protein pH 8, □ - 5 mM/g protein, pH 5.8.

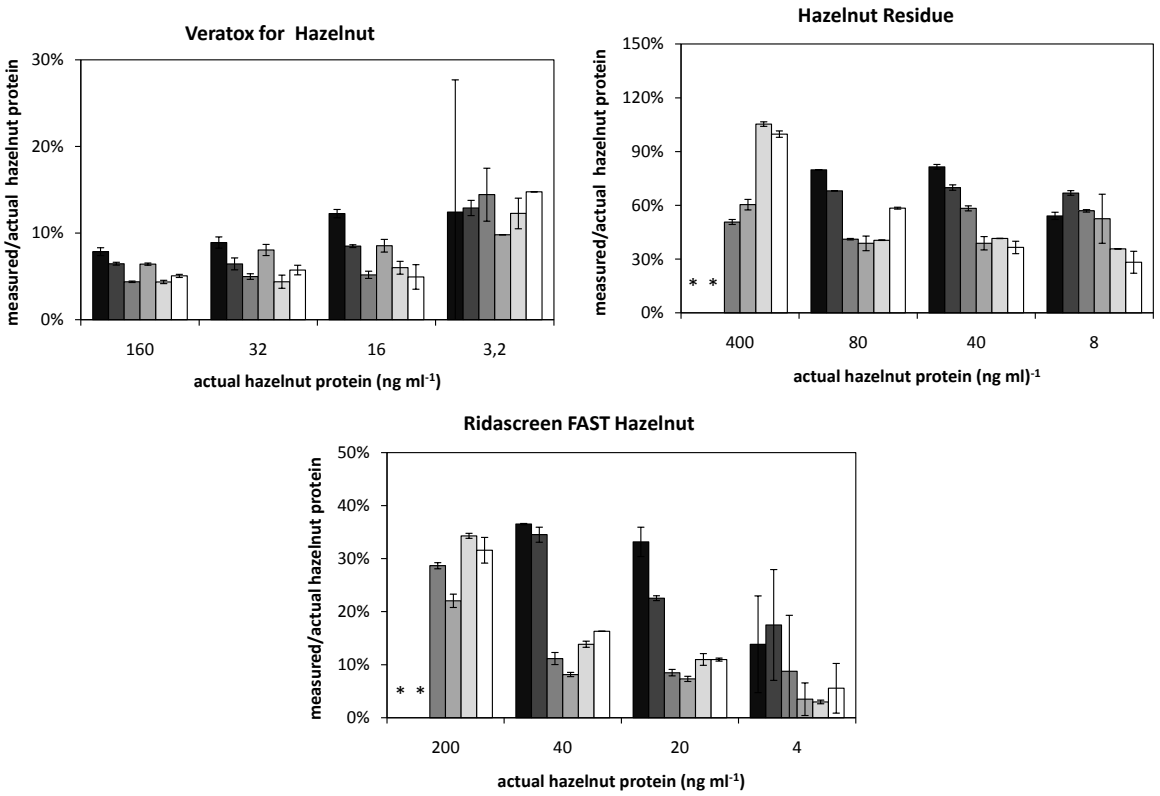


Figure 19 Ratio of measured over actual hazelnut protein concentration (y-axis) in different ELISA kits after duplicate analysis of a dilution series (x-axis) of a reference hazelnut sample and after heat incubation in the presence or absence of sunflower oil. ■ - 0 min, without oil, ■ - 0 min, with oil, ■ - 24 h, without oil, ■ - 24 h, with oil, □ - 48 h, without oil, □ - 48 h with oil

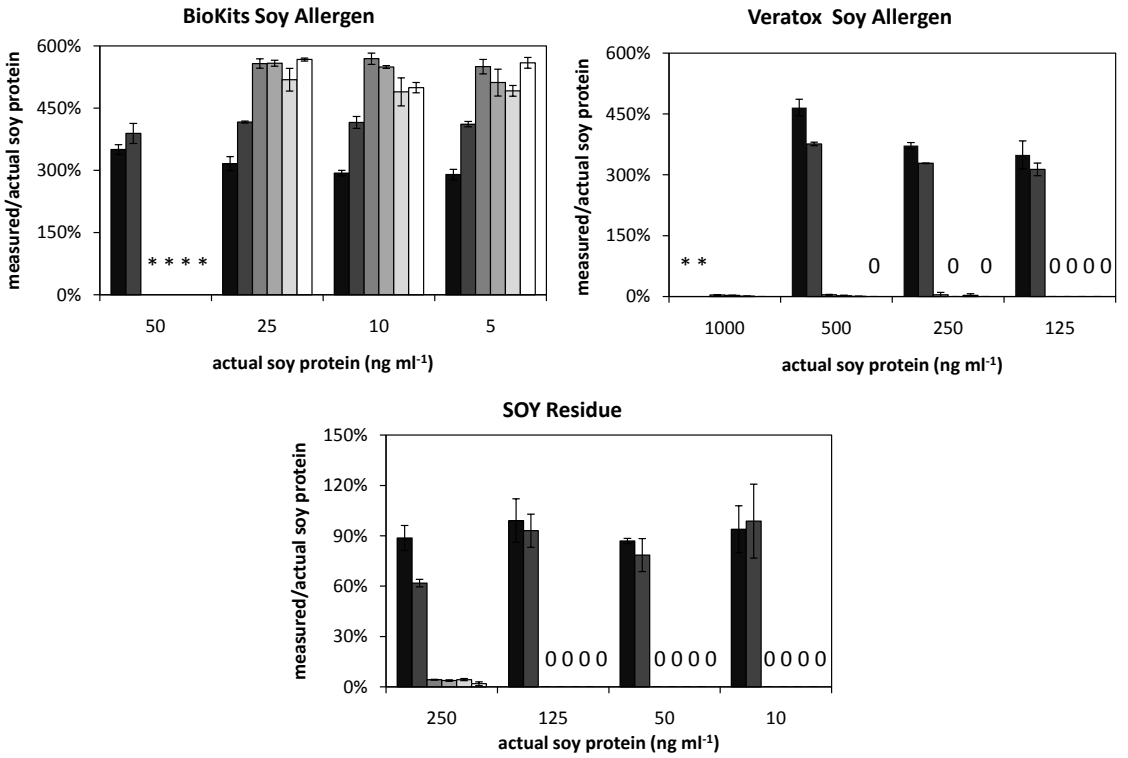


Figure 20 Ratio of measured over actual soybean protein concentration (y-axis) in different ELISA kits after duplicate analysis of a dilution series (x-axis) of a reference soybean sample and after heat incubation in the presence or absence of sunflower oil. ■ - 0 min, without oil, ■ - 0 min, with oil, ■ - 24 h, without oil, ■ - 24 h, with oil, □ - 48 h, without oil, □ - 48 h with oil

### Protein hydrolysis

Protein hydrolysis was performed using pepsin (Sigma Aldrich, 3200 units) at 37°C using a 1:250 enzyme/protein ratio (w/w) in the PBS buffer adjusted to pH 2. At different sampling points, the hydrolysis was stopped by increasing the pH to 6 using a 5 M NaOH solution.

Pepsin was used as a model enzyme for the hydrolysis. Trypsin could not be used for this purpose due to the presence of some trypsin inhibitors among the soybean proteins (such as Kunitz and Bowman Birk inhibitors) which would have hindered an effective peptide cleavage.

As a result of the induced hydrolysis, an increase in the non protein nitrogen and free amino groups was observed (Figure 21). The native hazelnut proteins contain about 8% of non-protein nitrogen and showed a 4-fold increase after 180 min of hydrolysis. On the contrary, the native soybean proteins contain about 6% of non-protein nitrogen and after 180 min of hydrolysis only a 2-fold increase was observed. These results are in accordance with the results obtained from the SDS-PAGE pattern.

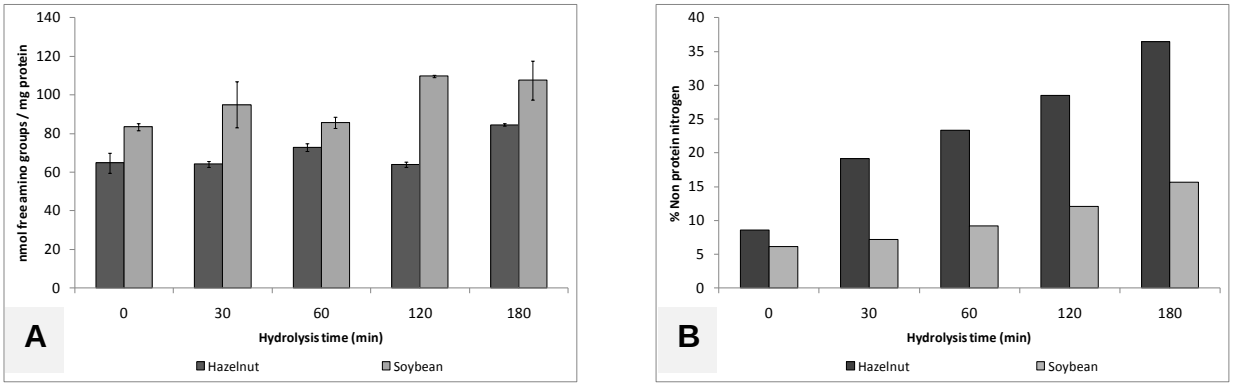


Figure 21 Increase in free amino group (A) and the non protein nitrogen fraction (B) as a result of the partial peptic hydrolysis

Soybean proteins turned to be fairly stable towards the induced hydrolysis. The 11S glycinin fraction (acid and basic subunit with the mass of 19 and 32 kDa, respectively) seemed to be especially affected by the peptic hydrolysis. On the other hand, the 7S conglycinin, especially the α' and β subunits, was fairly stable even after 180 min of hydrolysis (Figure 22 A). The degradation of the α subunit of the 7S conglycinin started only after 60 min of hydrolysis. Further, hydrolysis took place faster for the hazelnut proteins as manifested by the losses of intensity of the main protein bands with formation of peptides with masses between 10 and 15 kDA which is in agreement with previous literature data (Vieths *et al.*, 1999). The degradation of the main protein bands took place as soon as after 30 min of hydrolysis (Figure 22 B). Moreover, the presence of smaller peptides generated which were below the separation range of the used polyacrylamide gels cannot be excluded.

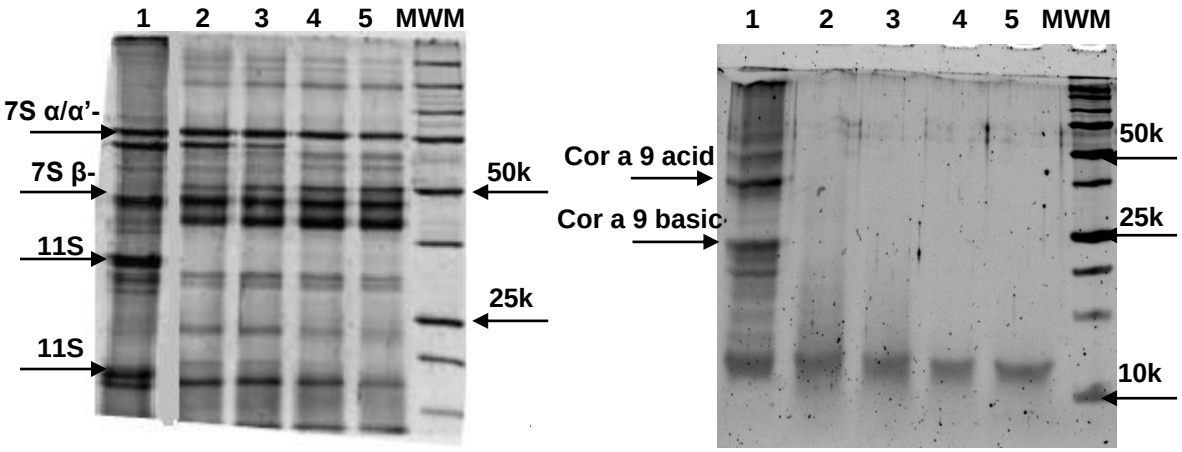


Figure 22 SDS-PAGE pattern of pepsin hydrolyzed soybean (A) and hazelnut protein isolates (B). Lane 1 - non hydrolyzed protein; lane 2-5 hydrolyzed for 30, 60, 120 and 180 min respectively; MWM - molecular weight marker

Analysis of both the reference and hydrolyzed samples with the BioKits Hazelnut assay returned absorbance values that were below the calibration range, similarly as for the lipid-induced oxidation model system. The only explanation the authors can currently give for this observation is that possibly some step in the sample preparation was interfering with the antibody binding in the assay.

As can be seen in Figure 23 the actual hazelnut protein concentration in the reference sample was underestimated with the Veratox for Hazelnut and Ridascreen FAST Hazelnut assay. With the Hazelnut Residue kit the actual hazelnut protein concentration measurement was concentration dependent, with an underestimation in the higher concentration range (800 and 400 ng/mL) and an overestimation in the lower concentration range (80 and 40 ng/mL).

Upon hydrolysis the hazelnut protein detection by the Hazelnut Residue kit proved to be quite stable, although the detection was reduced after 180 min. An increased detection of the hazelnut proteins after hydrolysis was recorded with the Veratox for Hazelnut. No further significant impact of the hydrolysis on the detectability was however observed after prolonged incubation of the samples. In the case of the Ridascreen Fast Hazelnut kit the detectability of the hazelnut proteins was severely affected by as little as 30 min of hydrolysis with an (almost) complete disruption of the hazelnut protein detection.

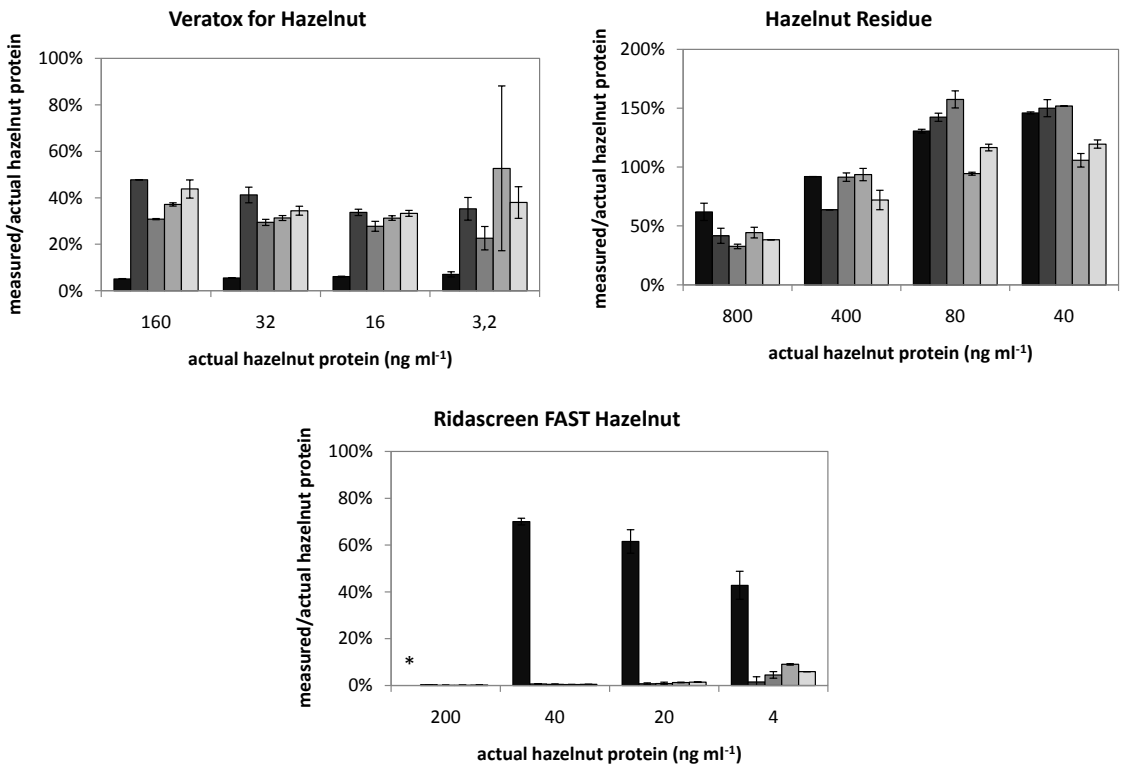


Figure 23 Ratio of measured over actual hazelnut protein concentration (Y-axis) in different commercial ELISA kits after duplicate analysis of a dilution series (X-axis) of a reference hazelnut protein sample and after protein hydrolysis by incubation with pepsin. ■ - reference sample, ■ - 30 min, ■ - 60 min, ■ -120 min, □ - 180 min (* = absorbance value of sample outside the range of the standard curve)

The soybean protein concentration measured in the untreated reference samples was overestimated with the Veratox Soy Allergen and the BioKits Soy Allergen assays, respectively and fairly estimated with the Soy Residue kit (Figure 24). With the BioKits Soy Allergen assay the detection decreased after 30 min of peptic hydrolysis, however, it was recovered again upon prolonged incubation. After 180 min an overall decreased detection was recorded with eventually only about 50 % of the soybean proteins being detected. In the case of the Veratox Soy Allergen, similarly as with the Veratox for Hazelnut assay, an increase in the detectability was observed after 30 min of hydrolysis, however, the detection decreased gradually as the hydrolysis progressed. A decrease in the detectability of about 50 % was observed after 180 min of incubation as compared to the native soybean extract. A similar progressive decrease of the soybean protein detection upon pepsin incubation was observed with the Soy Residue kit, although to a more severe extent as compared to the Veratox Soy Allergen kit. After 180 min of hydrolysis only about 20 % of the soybean protein could be detected.

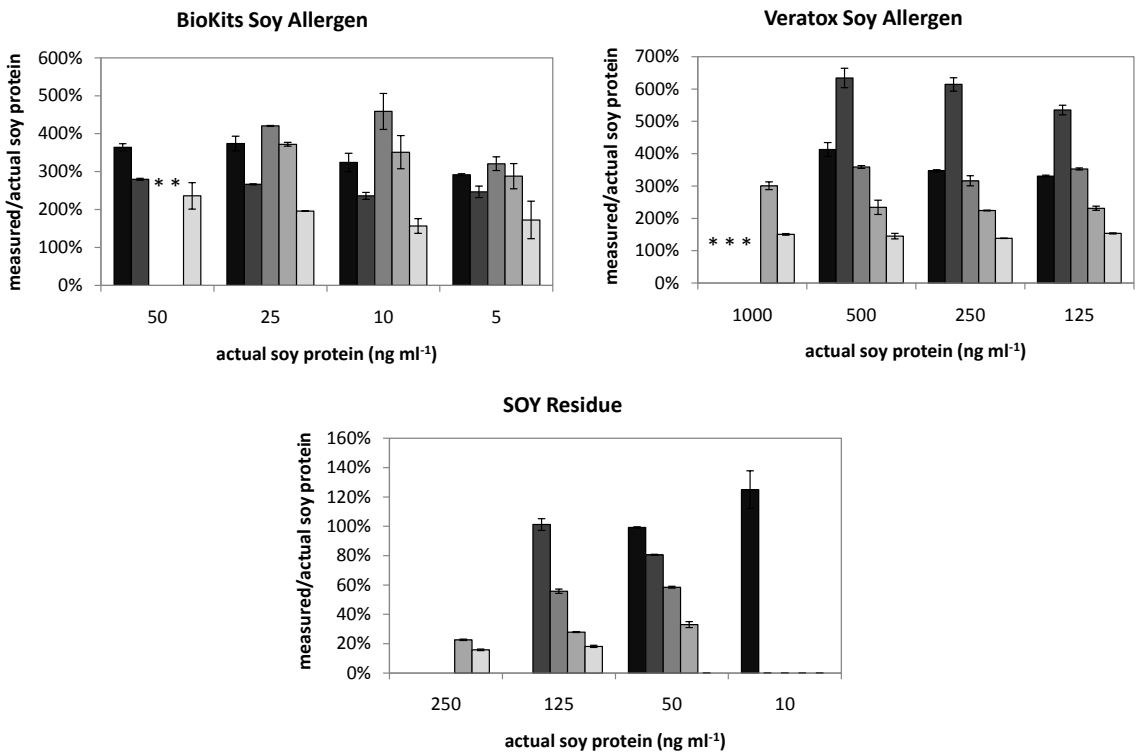


Figure 24 Ratio of measured over actual soybean protein concentration (Y-axis) in different ELISA kits after duplicate analysis of a dilution series (X-axis) of a reference soybean protein sample (darkest) and after protein hydrolysis by incubation with pepsin. ■ - reference sample, ■ - 30 min, ■ - 60 min, ■ -120 min, □ - 180 min (* = absorbance value of sample outside the range of the standard curve)

### 2.2.4 Detection of hazelnut in food model systems by ELISA and PCR

The work presented in the previous section has investigated the influence of food processing on the detection of hazelnut and soybean proteins by means of ELISA by inducing chemical modifications in buffered model systems. In this section this investigation is taken to a next level being more representative for real-life situations by studying model food systems. The idea behind the experimental set-up was to define to what extent hazelnut could be traced down after contaminating wheat flour during the production process of cookies. To this end wheat flour was incurred with different concentrations of defatted hazelnut powder. Defatting was done to facilitate homogenous distribution of the hazelnut powder in the flour. The incurred flour was then used to prepare other matrices, which represent different phases in the cookie production process. The different food samples where then analysed by means of ELISA and real-time PCR to elucidate the applicability of both detection platforms for the detection of hazelnut in processed food products.

To prepare the different food model systems wheat flour was incurred with the defatted hazelnut powder. This incurred flour was subsequently used to prepare the other matrices, as depicted in Figure 25. The composition of the model systems (Figure 25) is based on the cookie recipe of the AACC 10-50.05 method used to

assess the bake quality of cookie flour (American Association of Cereal Chemists, 2000).

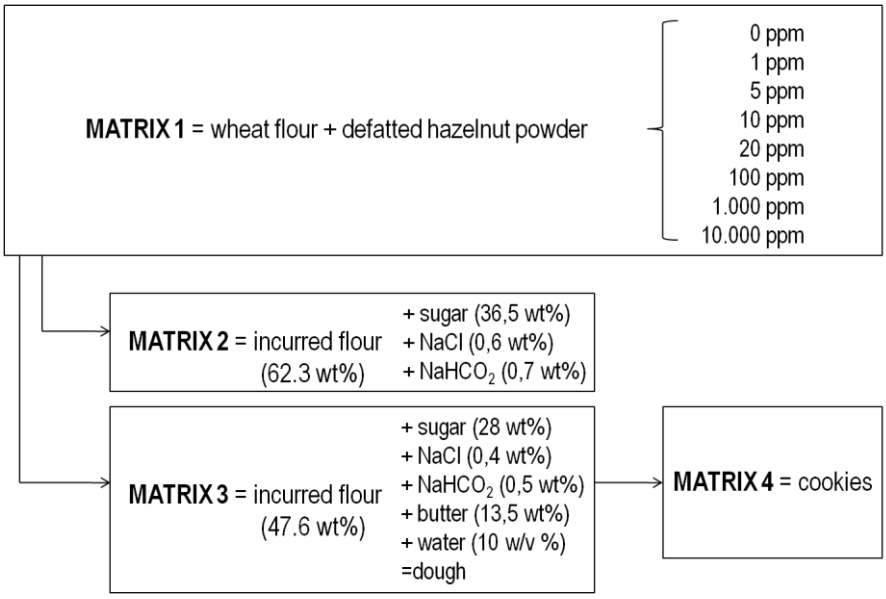


Figure 25 Schematic representation of the preparation of the different food model systems

The highest incurred level of 10.000 ppm was prepared by adding 10 g of defatted hazelnut powder to 990 g of wheat flour. The components were mixed thoroughly in a Kenwood kitchen mixer for 1 h at level 2 and intermediate scraping of the mixing bowl every 10 min. Before diluting the 10.000 ppm flour to prepare the other incurred levels, the homogeneity of the batch was verified. To this end, 9 samples of 100 mg were taken from the 10.000 ppm batch and DNA was extracted with the Qiagen DNeasy Plant mini kit. Samples were analysed with the *Cor a* 8 PCR assay in triplicate. No significant difference could be found between the mean $C_q$ values of the different samples (data not shown), indicating that a homogeneous incurrence was obtained and that the applied mixing procedure could be followed to prepare the further dilutions. The other incurred levels were prepared by serial dilution of the 10.000 ppm incurred flour with 'blank' wheat flour through mixing as described above.

Matrix 2 was prepared by adding the sugar, NaCl and $NaHCO_3$ to the flour in the bowl and the components were mixed for 3 min at level 2 with scraping of the bowl every minute. For the cookie dough (matrix 3) the butter was mixed with the sugar, NaCl and $NaHCO_3$ during 3 min at level 2 and the mixture was scraped down every min. Then the water was added and mixed with the other components for 1 min at level 1. After scraping the mixture down it was mixed for another minute at level 2. Finally, the (spiked) flour was added to complete the dough and mixed with the rest for 2 min at level 1 and scraped down every 30 s. The dough was divided in two: one part was used to analyse as such and the other part was used to prepare matrix 4.

The cookies were baked at 180°C for 16 min and the weight was determined before and after baking to determine the weight loss.

Those kits that performed best in the previous experiments were chosen for this study, i.e. the Ridascreen FAST Hazelnut and the BioKits Hazelnut assay. We selected the matrices incurred or prepared with the flour incurred with 0, 1, 5, 10, 20 and 100 ppm defatted hazelnut powder, which are concentrations within or just above the calibration range of the ELISA kits used.

For each matrix identical sample portions were analysed. However, by adding other components to the incurred wheat flour to prepare the other matrices or baking the dough, the concentration of the incurred hazelnut powder will be different in the different matrices. These factors were taken into account with respect to the interpretation of the obtained results. By considering the weight percentage of the incurred flour in the different matrices (see Figure 25), the amount of hazelnut detected is normalised to the corresponding amount detected in 100 % incurred flour. An additional conversion had to be made because the matrices were incurred with defatted hazelnut powder, a more concentrated form of hazelnut, the latter being the reporting unit of the kits' standards.

All incurred samples analysed returned positive results, i.e. absorbance measurements higher than that obtained for the zero standard of the respective ELISA kit. No cross-reactivity of the flour in the blank samples of matrix 1 was seen, as already been proven in the specificity analysis of the ELISA assays (2.2.1). However, the blank samples of matrix 2 returned increased absorbance values compared to noise signal of matrix 1. Quantification of the measured signal assumed that these samples contained 0.61±0.27 ppm and 0.98±0.26 ppm hazelnut, as determined with the Ridascreen FAST and BioKits Hazelnut assay, respectively. The signal decreased again for the samples of matrix 3 (0.26 ± 0.02 ppm and 0.18 ± 0.02 ppm, respectively) and 4 (0.23±0.01 ppm and 0.14±0.04 ppm, respectively), possibly due to dilution with the other matrix components, but still remaining higher than the noise. This indicates that the salts present in matrix 2 have an impact on the performance of both ELISA kits. The sugar present in matrix 2 is not contributing to the signal as also been proven already (2.2.1). Altered antigen-antibody binding at increasing ionic strengths has been reported previously (De Meulenaer *et al.*, 2005a), which could explain our observations. This background signal will however also be contained within the signal obtained with the spiked samples and will lead to a wrong quantification of the actual amount of hazelnut present. As this study intends to evaluate the impact of processing on the detection of the hazelnut proteins, the background detection of each matrix was subtracted from the respective samples in order to eliminate contributing signals not originating from the hazelnut proteins,.

With the Ridascreen Fast Hazelnut kit the absorbance values measured in the flour incurred with 10 ppm and higher concentrations of defatted hazelnut flour were above the calibration range. This is not surprising, as the actual hazelnut concentration in these samples is about 26 ppm or higher, taken into account the defatting, and the highest point in the calibration curve is 20 ppm hazelnut. In matrix 1 there is a good quantification of the actual hazelnut concentration, indicated by the ratio of on average 101 ± 13 % in the samples containing 5 ppm hazelnut (Figure 26, Left). In the samples containing 1 ppm hazelnut it seemed more difficult to correctly quantify the hazelnut concentration, which could be due to the low incurrence level, pushing the limits of the kit having an quantification limit of 2,5 ppm hazelnut as indicated by the manual. Upon analysis of the samples of matrix 2 the detected amount of hazelnut was decreased with on average about 35 %. This decreased detection could be explained by a negative influence of the salts, as mentioned earlier, and possibly sugar present. Although the sugar does not contribute to the background signal as explained above, it could have a negative impact on the protein-antibody interaction.. The addition of butter present in matrix 3 did not seem to have a further impact on the detection. The influence of the food matrix on allergen detection has been reported earlier by Whitaker *et al.* (2005), who saw a difference in the amount of peanut proteins that could be detected with three commercial ELISA kits in different food matrices. An additional decrease of the detected amount of hazelnut with on average about 10 % could be seen upon analysis of the samples of matrix 4. This indicates that apart from the matrix impact, an influence of the baking process could also be observed.

Similarly as for Ridascreen FAST kit, quantification of the hazelnut in the flour (matrix 1) with the BioKits Hazelnut assay was possible up to a level of 10 ppm as the values obtained for the higher concentrations were outside the calibration range. An overestimation of about 30 % on average was seen for the quantification of hazelnut in the samples of matrix 1 (Figure 26, Right). Although the background signal measured in the blank samples of matrix 2 was higher compared to the Ridascreen FAST assay, the addition of the salts and sugar did not seem to have a substantial impact on the hazelnut detection. On the contrary, a major impact of the baking process on the detection can be seen. The amount of hazelnut that was quantified in the cookies was on average only 17 % of the quantity measured in the flour, while this was about 55 % with the Ridascreen FAST kit.

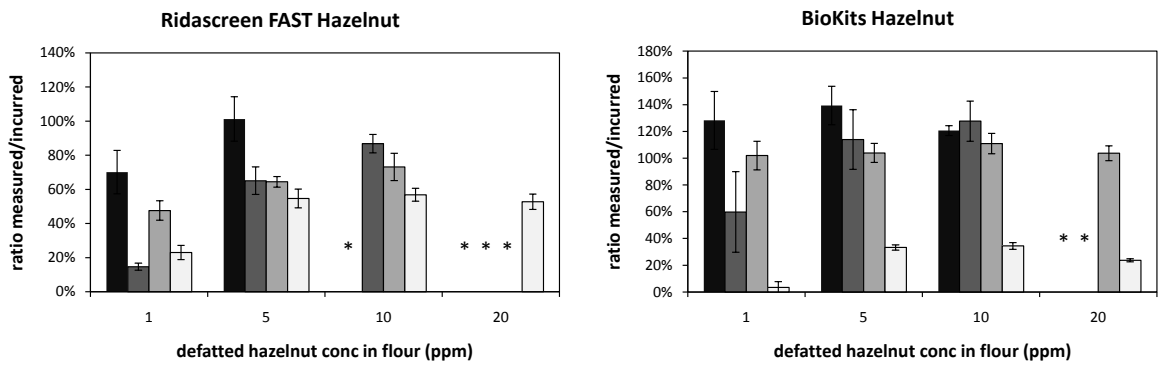


Figure 26 ELISA detection of hazelnut in different food model systems (Left) Ridascreen FAST Hazelnut (Right) BioKits Hazelnut Assay ■ - matrix 1 (wheat flour), ■ - matrix 2 (wheat flour + sugar, NaCl and $NaHCO_3$, ■ - matrix 3 (cookie dough), □ - matrix 4 (cookies). Data are mean values of six samples analysed in duplicate.

After DNA extraction from the different food samples, the latter were analysed in the Cor a 8, First Hazelnut and Surefood Hazelnut PCR. In each PCR run a dilution series of genomic DNA of defatted hazelnut powder was analysed in parallel to construct a calibration curve. Based on this calibration curve the amount of DNA was calculated from the obtained $C_q$ values of the samples. Similarly as described above for the ELISA-based analysis, the altered concentration of the defatted hazelnut powder arising from addition of the other components to the flour to prepare matrix 2 to 3 and the loss of weight due to baking the cookies (matrix 4) has been taken into account.

Analysis of the samples of matrix 1 (incurred flour) with the Cor a 8, the Surefood Hazelnut and the First Hazelnut PCR resulted in an amplification curve for all twelve replicates starting from 100 ppm. At the 10 ppm only 4, 6 and 11 of the 12 replicates, respectively, showed an amplification curve and the rest wad undetected. Only the data points of the concentrations where all replicates were positive have been included in Figure 27.

When comparing the amount of DNA that was detected in all the samples with the different assays, the values obtained with the Cor a 8 PCR are lower than those obtained with the Surefood Hazelnut and the First Hazelnut PCR (Figure 27). The latter two returned comparable values. Very surprisingly, the amount of DNA that is detected in the samples of matrix 2 is higher than the amount in matrix 1, and a further increase in the detection is seen in the samples of matrix 3. After baking, a decreased detection is observed. The observed trend is visible for the three PCR assays, indicating that the cause is inherent to the samples, although it is more pronounced in the Cor a 8 PCR and less in the First Hazelnut PCR.

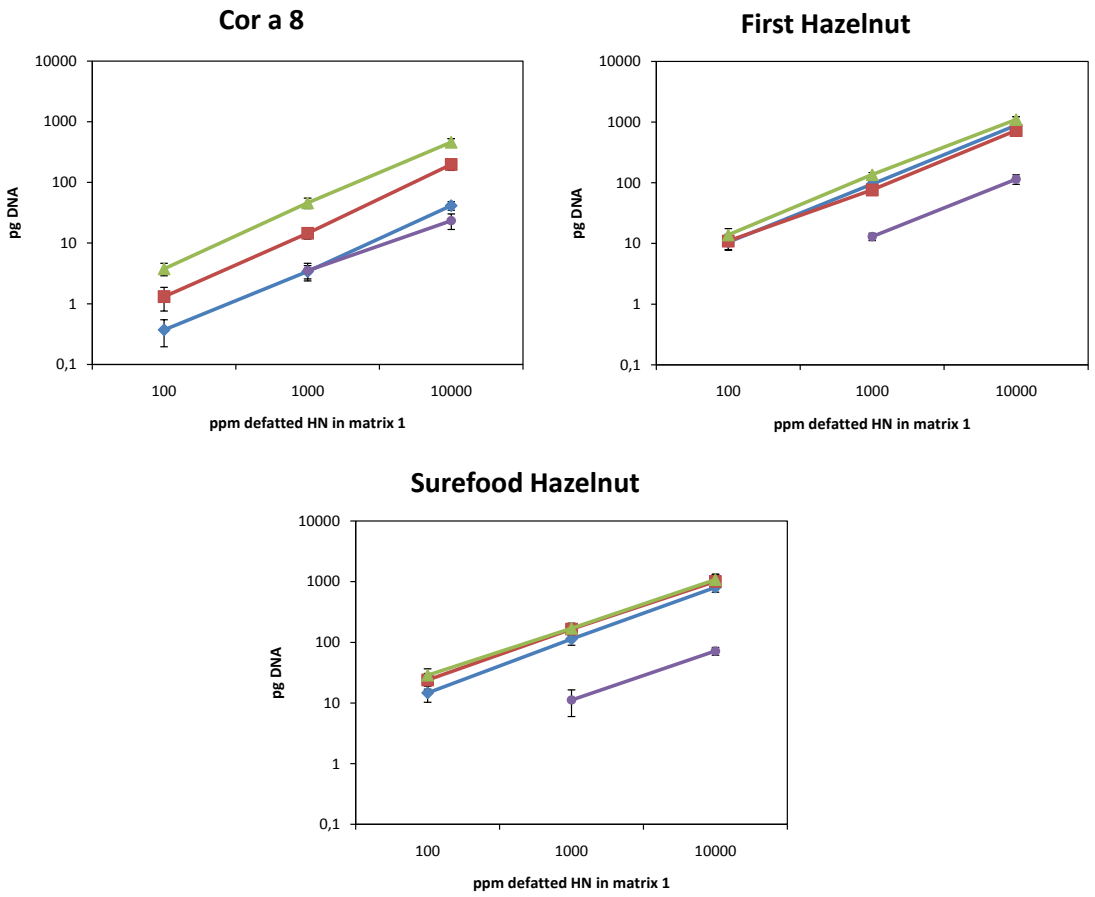


Figure 27 Amount of hazelnut DNA detected with the distinct PCR assays in different food matrices (blue-matrix 1, red-matrix 2, green-matrix 3, purple-matrix 4) incurred with defatted hazelnut powder at 100, 1000 and 10000 ppm. Data are mean values of 6 replicates analysed in duplicate ± SD

One hypothesis was that more DNA was extracted from the samples of matrix 2 and 3. The reasoning behind it was that possibly the wheat flour alone did not suspend completely in the volume of extraction buffer used, although attention was paid to this during the extraction procedure. In the other matrices, the weight percentage of the wheat flour is lower, making it easier to suspend and promoting DNA extraction. To confirm this hypothesis, the DNA content of the samples was measured. The obtained DNA concentration comprised the amount of DNA from the defatted hazelnut flour and wheat flour. As only minor amounts of defatted hazelnut powder are present compared to the majority of wheat flour, no big differences concerning the DNA content between samples of the different incurrence levels are expected. The other components that were used to prepare the food samples do not contain DNA. The concentrations were normalised taken into account the different weight percentages of the (incurred) wheat flour in the different matrices. Figure 28 shows that the proposed hypothesis is not valid as the DNA concentration in the samples of matrix 2 and 3 is slightly lower than that of the matrix 1 samples. The lower DNA content in the cookie samples is consistent with the data obtained by

PCR. However, it remains unclear whether the decreased DNA concentration in the cookie compared to the dough is resulting from DNA degradation or reduced extractability due to the baking process.

Based on the observations it could be concluded that some components present in matrix 2 and 3 that have not been removed during DNA extraction are positively influencing the PCR reaction, leading to an increased detection. Alternatively, it seems more plausible that components present in the wheat flour inhibit the PCR reaction, with the concentration of inhibitors being lower in matrix 2 and 3 compared to matrix 1.

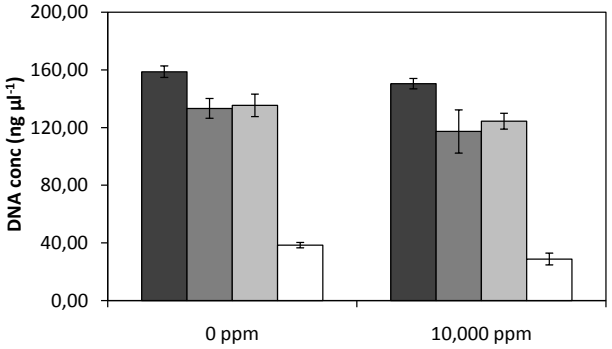


Figure 28 Normalised DNA concentration in the samples of the different matrices prepared with blank wheat flour (0 ppm) and incurred wheat flour (10000 ppm) ■ - matrix 1, ■ - matrix 2, ■ - matrix 3, □ - matrix 4. Data are mean values of duplicate measurements of six replicates with standard deviation.

Although the *Cor a* 8 PCR assay was the least sensitive, producing the lowest number of positive amplification reactions at the lower concentrations, it had the highest recovery upon analysis of the cookie samples. For the *Cor a* 8 assay 53 % of the amount of genomic hazelnut DNA detected in the incurred flour could still be detected in the cookies, while this was only 9 % and 13 % for the Surefood Hazelnut and First Hazelnut PCR assays, respectively.

From these experiments it can be concluded that the investigated ELISA procedures were able to detect the incurred hazelnut to a lower level (1ppm defatted hazelnut) in the different matrices than the investigated real-time PCR procedures (100 ppm defatted hazelnut). The lower sensitivity measured for the PCR's is not necessarily inherent to assays, but could also result from a sub-optimal DNA extraction procedure. However, the applied DNA extraction procedure is used successfully for routine GMO analysis on food and feed. Nonetheless, it seems interesting to investigate if other DNA isolation procedures which result in a higher yield and equal purity than the Qiagen DNeasy Plant mini kit, could aid in improving the sensitivity of the PCR-based detection.

A negative matrix effect on the hazelnut detection was observed in one of the two ELISA assays. The matrix also seemed to have a negative influence on the DNA amplification, a trend being independent from the type of PCR assay investigated. A common characteristic of both detection platforms is their susceptibility to food processing on the detection of hazelnut in the cookies. However, from this study we cannot conclude whether or not DNA is less susceptible to food processing than proteins. A semi-quantitative comparison between both detection platforms can be made based on the recovery of the amount of hazelnut (DNA) detected in the incurred flour and cookies respectively. However, as the recovered amount varies considerably between the different assays for both types of detection methods, no unequivocal conclusion can be made.

## 2.3 Mass-spectrometric study of changes in allergen proteins by application of controlled chemical reaction

Mass spectrometry is one of the dominant approaches used for the identification of proteins and peptides. Given that food allergens are mainly proteins, mass spectrometry is gaining more and more attention in the context of the food allergens detection. The use of mass spectrometric approaches for the identification of specific highly stable peptides as markers for the presence of peanut allergens was previously described (Shefcheck *et al.*, 2006; Shefcheck and Musser, 2004). However, reliable stable peptide markers were found for only peanut and caseins so far. Therefore, efforts should be devoted to the discovery of stable peptide markers derived from other major food allergens as well, in order to develop reliable mass spectrometric methods (Careri *et al.*, 2007; Monaci and Visconti, 2009a). Screening of stable allergen derived peptides is especially important when complex matrixes are analyzed via mass spectrometric techniques where the selection of good peptide markers plays a critical role. Moreover, the target peptide must be unique to the proteins of interest.

Attempts to identify stable peptide markers from hazelnut and soybean were made. Following methodology was used for this. An aliquot of 60 µg of total protein were digested with trypsin at 37°C in 50 mM ammonium bicarbonate pH 7.8 at a protease to protein ratio of 1:48 (w/w). Peptides were concentrated by vacuum centrifugation and finally redissolved in 100 µL of 0.1 % formic acid. Digested peptides were first separated using the Ettan LC system equipped with an UV detector from GE Healthcare (Brussels, Belgium). The RP column was a 220 x 2.1 mm Spheri-5 PTC column with particle size of 5 µm from Higgins Analytical Inc. (Mountain View, California, USA) on which the peptides were loaded. The column was equilibrated with $H_2O$ 100% with 0.1% trifluoroacetic acid (TFA) and elution was

achieved by the following step gradient with acetonitrile 100% with 0,1% TFA. The flow rate was 0.1 mL/min and fractions of 0.2 mL were collected. The eluted peptides were monitored at 220 and 280 nm. The collected fractions were dried and redissolved in 10 µL of 0.1% formic acid. Finally, 1 µL of this peptide fractions was co-crystallized with 1 µL of α-cyano-4-hydroxycinnamic acid (α-CHCA) as matrix (5 mg of α-CHCA in 1 mL of 50% acetonitrile in 0.2% TFA). The Applied Biosystems MDS Sciex 4800 Plus MALDI TOF/TOF (Foster City, California, USA) operating in reflectron positive ion mode was used for the peptide detection. External calibration was performed with a mix of des-Arg$^1$-Bradykinin, Angiotensin I, Glu$^1$-Fibrinopeptide B, ACTH (1-17 clip), ACTH (18-39 clip), ACTH (7-38 clip) (Applied Biosystems 4700 proteomic analyzer Mass Standards Kit). Totals of 2000 shots were accumulated for each MS and MS/MS spectrum respectively. Protein identification was performed by searching in the protein sequence database (National Center for Biotechnology Information or SwissProt) using the Mascot search engine (http://www.matrixscience.com). The following search parameters were used: taxonomy – *viridiplantae,* precursor mass tolerance = 100 ppm; fragment mass tolerance = 0.5 Da. The enzyme entry was set for trypsin, the maximum number of missed cleavages was set to 2 and oxidized methionine, tryptophan and histidine, deamidation (Gln and Asn) and Glu ->pyro – Glu modification were considered as peptide modification. For the direct MALDI-TOF MS and MS/MS, 6 µg of the protein mixture were digested with trypsin (1:48 enzyme to protein ratio). The obtained peptides, vacuum dried, were redissolved in 10 µL HCOOH 0.1% and 1 µL of it was directly spotted on the MALDI plate together with 1 µL of α-CHCA matrix.

Peptides with masses below 950 Da can be hardly identified using the MALDI-TOF MS due to interferences caused by the matrix. Therefore the peptides were identified within a mass range of 950 to 4000 Da. Although hazelnuts contain a large number of proteins, peptides from mainly 3 proteins were found in the digest of the native hazelnut extract. These were mainly the 2S albumin (*Cor a 14*), 7S vicilin (*Cor a 11*) and the 11S globulin (*Cor a 9*) known as major hazelnut allergens. For soybean, peptides derived from mainly β-conglycinin (*Gly m 5*), glycinin (*Gly m 6*) and trypsin inhibitors were found.

After treatment under the food processing simulating reactions, the proteins were subjected to a number of molecular changes characterized by modification of amino acids residues, formation of protein bound carbonyls and changes in the electrophoretic pattern (3.2). It was therefore expected that after subjecting the proteins to the food processing simulating reactions, a number of the peptides previously detected in the native protein will not be detectable anymore. Despite this, a large number of the peptides remained stable in the tryptic digest of the modified proteins as well (Table X-Table XII). It seems however, that the lipid induced

oxidation had a most severe impact on the stability of the peptides derived from either hazelnut or soybean proteins. The reason can be either due to severe aggregation (Figure 16) which hindered an effective digestion by the trypsin either due to the observed lysine residues losses which are moreover the cleavage sites of the trypsin. The stable peptides derived from hazelnut and soybean allergens (especially major *Cor a 9* and *Gly m 5* and *Gly m 6*) can be further used for the development of reliable quantitative mass spectrometric methods. MALDI-TOF MS represents an excellent technique for fast and efficient screening of such stable peptides during food processing simulating reactions (Table X-Table XII).

Table X Identified peptides derived from the modified hazelnut proteins with their corresponding assigned scores as determined by MALDI-TOF MS and MS/MS analysis. Proteins were digested and the obtained peptides were separated on a RP column prior the MALDI-TOF MS analysis

| Expected $[M+H]^+$ | Peptide | Measured mass $[M+H]^+$ | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1% hazelnut [a] | 0.5% hazelnut 0.5% wheat [a] | 0.25% hazelnut 0.75% wheat[a] | 2.5 mmol HOCl/g | 5 mmol HOCl/g | Oxidized soybean oil [b] |
| | | | | **2S albumin – Cor a 14** | | | |
| 1131.53 | $^{54}$Gln-Arg$^{62}$ | 1131.50 | 1131.44 | 1131.43 (40) | 1131.45 | n/d | 1131.46 |
| 1305.60 | $^{105}$Gln-Arg$^{115}$ | 1305.57 (70) | 1305.50 (99) | 1305.48 (82) | 1305.48 | 1305.55 | n/d |
| | | | | **7S vicilin – Cor a 11** | | | |
| 1046.56 | $^{407}$Glu-Arg$^{415}$ | 1046.54 | 1046.48 | 1046.47 | 1046.48 | 1046.35 (38) | n/d |
| 1048.58 | $^{92}$Leu-Arg$^{100}$ | 1048.55 (44) | 1048.50 (55) | 1048.49 (37) | 1048.49 | 1048.56 (26) | n/d |
| **1077.53** | **$^{395}$Gly-Arg$^{403}$** | **1077.50 (58)** | **1077.44 (51)** | **1077.44 (47)** | **1077.45 (37)** | **1077.51 (50)** | **1077.43** |
| 1157.54 | $^{269}$His-Arg$^{278}$ | 1157.51 | 1157.62 | 1157.59 | n/d | n/d | n/d |
| 1202.53 | $^{305}$Gly-Arg$^{315}$ | 1202.50 | 1202.45 | 1202.42 | n/d | 1202.53 | 1202.49 |
| 1220.60 | $^{241}$Ala-Arg$^{251}$ | 1220.57 (48) | 1220.50 (57) | 1220.49 (52) | 12220.51 | 1220.58 | n/d |
| 1224.56 | $^{279}$Leu-Lys$^{288}$ | n/d | 1224.47 | 1224.45 | n/d | n/d | n/d |
| 1233.70 | $^{80}$Val-Arg$^{89}$ | 1233.66 | n/d | n/d | 1233.59 | 1233.68 (34) | n/d |
| 1331.71 | $^{153}$Ile-Arg$^{164}$ | 1331.68 | 1331.61 (25) | 1331.59 (22) | 1331.55 | n/d | n/d |
| 1363.73 | $^{202}$Ala-Lys$^{213}$ | 1363.69 (82) | 1363.63 | 1363.60 | n/d | n/d | n/d |
| **1415.70** | **$^{141}$Glu-Arg$^{152}$** | **1415.66 (95)** | **1415.58 (103)** | **1415.57 (62)** | **1415.54** | **1415.68 (33)** | **1415.60** |
| 1509.71 | $^{423}$Asn-Lys$^{435}$ | 1509.69 | n/d | n/d | n/d | 1509.69 | n/d |
| 1851.98 | $^{252}$Ile-Lys$^{268}$ | 1851.94 | 1851.86 | 1851.79 | n/d | n/d | n/d |
| 2182.93 | $^{56}$Glu-Arg$^{72}$ | n/d | 2182.91 | 2182.88 | 2182.77 | n/d | n/d |
| 2963.60 | $^{101}$Leu-Lys$^{127}$ | 2963.55 | 2963.53 | n/d | n/d | n/d | n/d |
| | | | | **11S globulin – Cor a 9** | | | |
| 996.59 | $^{280}$Leu-Arg$^{287}$ | 996.56 | 996.50 | 996.52 | n/d | 996.58 | n/d |
| 1002.54 | $^{364}$Trp-Arg$^{371}$ | 1002.51 (66) | 1002.45 (67) | 1002.45 (62) | n/d | n/d | 1002.45 |
| 1027.55 | $^{43}$Leu-Arg$^{51}$ | 1027.46 | 1027.47 | 1027.41 | 1027.48 | 1027.54 (45) | 1027.45 |
| **1076.52** | **$^{209}$Gln-Arg$^{217}$** | **1076.49 (89)** | **1076.44 (74)** | **1076.43 (69)** | **1076.44 (50)** | **1076.50 (45)** | **1076.48 (79)** |
| 1151.60 | $^{341}$Ala-Arg$^{350}$ | 1151.54 (92) | 1151.49 (74) | 1151.47 (53) | 1151.48 | n/d | 1151.52 (88) |
| 1356.69 | $^{446}$Thr-Arg$^{458}$ | 1356.65 | 1356.59 (61) | 1356.56 | 1356.59 | 1356.67 (80) | n/d |
| 1394.70 | $^{339}$Ser-Arg$^{350}$ | 1394.66 | 1394.60 | 1394.58 | 1394.60 | n/d | 1394.63 (54) |
| 1399.65 | $^{434}$Ala-Lys$^{445}$ | 1399.63 | 1399.55 | 1399.53 | 1399.55 | n/d | 1399.51 |
| **1440.82** | **$^{351}$Ile-Arg$^{363}$** | **1440.79 (94)** | **1440.71 (70)** | **1440.69 (52)** | **1440.71 (52)** | **1440.80 (88)** | **n/d** |
| 1555.75 | $^{433}$Arg-Lys$^{445}$ | 1555.73 | 1555.74 | 1555.72 | 1555.59 | n/d | n/d |
| **1629.86** | **$^{464}$Ala-Arg$^{478}$** | **1629.83 (142)** | **1629.74 (76)** | **1629.72 (138)** | **1629.74 (117)** | **1629.84 (145)** | **n/d** |
| 1698.76 | $^{195}$His-Arg$^{208}$ | 1698.73 (106) | 1698.65 (74) | 1698.61 (108) | 1698.65 (51) | n/d | 1698.66 |
| **1934.91** | **$^{401}$Val-Arg$^{417}$** | **1934.87 (137)** | **1934.77 (129)** | **1934.74 (102)** | **1934.77 (113)** | **1934.89 (164)** | **1935.81(153)[#]** |
| 2338.21 | $^{377}$Glu-Arg$^{396}$ | 2338.16 | n/d | n/d | n/d | n/d | n/d |
| 2643.40 | $^{80}$Thr-Arg$^{102}$ | 2643.35 (129) | 2644.10 (82)# | 2643.12 | 2643.23 (94) | n/d | n/d |

*[a] – 48h incubated with 6% glucose at 70°C; in parenthesis assigned score: >34 = significant homology; > 47 = identity or extensive homology (p<0.05); no parenthesis behind the precursor mass – no MS/MS performed due to lower abundance; n/d – not detected; underlined peptide masses - peptides with a missed cleavage; #- deamidation (Asn and Gln); shaded – stable peptides*

Table XI Identified peptides derived from the modified soybean proteins (β-conglycinin and the trypsin inhibitors) with their corresponding assigned scores as determined by MALDI-TOF MS and MS/MS analysis. Proteins were digested and the obtained peptides were separated on a RP column prior the MALDI-TOF MS analysis

| Expected $[M+H]^+$ | Peptide | Measured mass $[M+H]^+$ | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1% soybean [a] | 0.5% soybean 0.5% wheat [a] | 0.25% soybean 0.75% wheat [a] | 2.5 mmol HOCl/g | 5 mmol HOCl/g | Oxidized soybean oil [b] |
| | | **Gly m 5.0101 (α-chain)** | | | | | |
| **955.53** | **$^{224}$Ser-Arg$^{231}$** | **955.54 (61)** | **955.42 (51)** | **955.54 (47)** | **955.48 (57)** | **956.49 (14)§** | **955.51** |
| 1051.53 | $^{194}$Asn-Arg$^{202}$ | 1051.53 | 1051.46 (61) | 1051.55 | 1051.49 | 1051.52 | 1051.44 |
| 1079.55 | $^{410}$Ser-Lys$^{418}$ | 1079.55 (25) | 1079.54 (27) | 1079.57 (30) | 1079.47 | 1079.51 | n/d |
| 1084.46 | $^{113}$Gln-Arg$^{120}$ | 1084.49 (52) | 1084.48 (63) | 1084.50 (47) | 1084.38 | 1084.41 | n/d |
| 1188.52 | $^{361}$Glu-Arg$^{370}$ | 1188.55 | 1188.54 | 1188.54 | 1188.42 (48) | 1188.46 (36) | 1188.44 |
| 1378.74 | $^{152}$Gln-Lys$^{162}$ | 1378.74 (55)$ | 1378.61 (48) | 1378.74 (54) | 1378.68 | 1378.70 | 1378.61 |
| **1391.87** | **$^{306}$Leu-Arg$^{318}$** | **1391.91 (66)** | **1391.76 (43)** | **1391.74 (35)** | **1391.83** | **1391.85** | **1391.52** |
| 1406.73 | $^{398}$Thr-Arg$^{409}$ | 1406.73 (52) | 1406.60 (59) | 1406.73 (60) | 1406.70 | 1406.70 (40) | n/d |
| 1465.64 | $^{178}$Glu-Arg$^{189}$ | 1465.67 | 1465.65 | 1465.67 | 1465.67 (10) | 1465.71 | 1465.62 |
| 1534.82 | $^{397}$Lys-Arg$^{409}$ | 1534.82 (28) | 1534.67 | 1534.79 | 1534.76 (92) | 1534.79 (67) | 1534.67 |
| **1618.86** | **$^{422}$Phe-Arg$^{434}$** | **1618.90 (90)** | **1618.73** | **n/d** | **1618.79 (57)** | **1618.83 (82)** | **n/d** |
| 1770.99 | $^{371}$Leu-Arg$^{385}$ | n/d | 1770.86 | 1770.86 | 1770.94 (18) | 1770.83 (11) | n/d |
| 1792.92 | $^{152}$Gln-Arg$^{165}$ | 1792.73 | 1792.92 | n/d | 1792.85 (13) # | n/d | n/d |
| 1939.78 | $^{136}$Gly-Arg$^{151}$ | 1936.69 (122) | 1939.69 (154) | 1939.74 (128) | 1939.55 (106) | 1939.64 | n/d |
| **2025.95** | **$^{479}$Glu-Arg$^{494}$** | **2025.96 (148)** | **2025.74 (158)** | **2025.97 (138)** | **2025.85 (139)** | **2025.91 (123)** | **2025.81** |
| 2038.88 | $^{113}$Gln-Arg$^{127}$ | 2038.90 (68) | 2038.70 (77) | 2038.90 (42) | 2053.72 (16) # | n/d | n/d |
| **2152.03** | **$^{287}$Val-Arg$^{305}$** | **2152.04 (150)** | **2151.83 (147)** | **2152.04 (114)** | **2151.94 (169)** | **2151.99 (80)** | **n/d** |
| 2169.03 | $^{322}$Met-Arg$^{340}$ | n/d | n/d | n/d | n/d | 2170.95 (23)§ | n/d |
| 2253.84 | $^{133}$Gly-Arg$^{151}$ | n/d | 2253.88 | n/d | 2253.68 (109) | 2253.79 (100) | n/d |
| 2459.15 | $^{319}$Phe-Arg$^{339}$ | 2458.94 (142) | 2459.01 (190) | 2459.15 | 2459.07 (123) | 2459.09 | n/d |
| | | **Gly m 5.0201 (α'-chain)** | | | | | |
| 986.54 | $^{240}$Ser-Arg$^{247}$ | 986.54 (49) | 986.53 (40) | 986.55 (38) | 986.48 (47) | 986.51 | 986.57 |
| 1078.43 | $^{136}$Gly-Arg$^{144}$ | 1078.43 (37) | 1078.45 (51) | 1078.46 (42) | 1078.35 | 1078.40 | n/d |
| 1189.51 | $^{396}$Glu-Arg$^{405}$ | 1189.52 (76) | 1189.53 (63) | 1189.54 (39) | n/d | 1189.48 | n/d |
| **1383.69** | **$^{518}$Gln-Arg$^{528}$** | **1383.70 (77)** | **1366.52 (62)#** | **1383.70 (71)** | **1383.61 (36)** | **1383.66** | **1383.59 (91)** |
| 1390.71 | $^{145}$Glu-Lys$^{155}$ | 1390.73 (21) | 1390.71 (37) | 1390.74 (24) | 1390.80 | 1390.77 | 1390.44 |
| 1511.78 | $^{518}$Gln-Lys$^{529}$ | n/d | n/d | n/d | 1511.67 | 1511.70 (14) | 1512.63 (74)§ |
| 2422.06 | $^{110}$Gln-Arg$^{127}$ | 2422.07 | 2422.04 (63) | n/d | n/d | n/d | n/d |
| | | **Gly m 5.0301 (β-chain)** | | | | | |
| 956.52 | $^{63}$Ser-Arg$^{70}$ | 956.54 | 956.55 | 956.53 | 956.48 | 956.49 (15) | 956.41 |
| 1235.59 | $^{195}$Val-Arg$^{204}$ | 1235.60 | 1235.48 (60) | 1235.59 | n/d | 1235.57 (17) | n/d |
| 1256.61 | $^{320}$Gln-Arg$^{329}$ | 1256.63 | 1256.48 | 1256.63 | 1256.55 (29) | 1256.58 (34) | 1256.52 |
| 1361.72 | $^{371}$Asn-Arg$^{382}$ | 1361.73 (54) | 1361.62 | 1361.71 | 1361.67 | n/d | n/d |
| 1372.62 | $^{114}$Asp-Arg$^{125}$ | 1372.64 | 1372.62 (58) | 1372.65 (67) | n/d | n/d | n/d |
| 1407.68 | $^{232}$Thr-Arg$^{243}$ | 1497.72 | 1407.56 | 1407.55 | 1407.62 | 1407.66 (34) | 1407.56 |
| 1512.77 | $^{318}$Gln-Arg$^{329}$ | 1512.76 (46) | 1512.76 | 1512.80 (63) | 1512.68 | n/d | 1512.63 (92) |
| 1618.86 | $^{256}$Phe-Arg$^{268}$ | 1618.90 (90) | 1618.73 | n/d | 1618.79 (57) | 1618.83 (82) | n/d |
| 1773.88 | $^{387}$Gln-Arg$^{402}$ | 1773.92 (115) | 1773.73 (100) | 1773.88 | 1773.81 (21) | 1773.72 (31) | n/d |
| 2300.16 | $^{383}$Gln-Arg$^{402}$ | n/d | n/d | n/d | 2300.13 | 2286 (13)#§ | n/d |
| | | **7S globulin basic subunit** | | | | | |
| 1545.83 | $^{389}$Gln-Arg$^{401}$ | 1545.70 (82) | 1545.75 (93) | 1545.72 | 1528.66 (41)# | 1545.75 (48) | n/d |
| | | **Trypsin inhibitor A** | | | | | |
| 997.42 | $^{136}$Asp-Arg$^{143}$ | 997.31 | 997.35 (57) | 997.35 | n/d | n/d | n/d |
| 1163.64 | $^{77}$Gly-Arg$^{87}$ | 1163.67 (56) | 1163.54 (39) | 1163.53 | n/d | n/d | n/d |
| 1200.71 | $^{190}$Asn-Lys$^{199}$ | 1200.62 | 1200.60 | 1200.63 | 1200.65 (35) | 1201.59 (18)§ | n/d |
| 1230.53 | $^{147}$Val-Lys$^{156}$ | 1230.52 (45) | 1230.40 | 1230.54 (38) | 1230.46 (66) | 1230.50 | n/d |
| | | **Trypsin inhibitor B** | | | | | |
| 1200.71 | $^{166}$Asn-Lys$^{175}$ | 1200.62 | 1200.60 | 1200.63 | 1200.65 (35) | 1201.59 (18)§ | n/d |
| 1230.53 | $^{123}$Val-Lys$^{132}$ | 1230.52 (45) | 1230.40 | 1230.54 (38) | 1230.46 (66) | 1230.50 | n/d |
| | | **Kunitz trypsin inhibitor** | | | | | |
| 1200.71 | $^{188}$Asn-Lys$^{197}$ | 1200.62 | 1200.60 | 1200.63 | 1200.65 (35) | 1201.59 (18)§ | n/d |
| 1351.63 | $^{136}$Asp-Arg$^{146}$ | 1351.49 (85) | 1351.54 (91) | 1351.52 | n/d | n/d | n/d |

*[a] – 48h incubated with 6% glucose at 70°C; [b] – 48h incubated with 1% highly oxidized soybean oil at 70°C; in parenthesis assigned score: >13 = significant homology; > 26 = identity or extensive homology (p<0.05); no parenthesis behind the precursor mass – no MS/MS performed due to lower abundance; n/d – not detected; underlined peptide masses - peptides with a missed cleavage; # - Glu ->pyro Glu on the N terminal; §-deamidation (Asn and Gln); shaded – stable peptides*

Table XII Identified peptides derived from the modified soybean proteins (glycinin fraction) with their corresponding assigned scores as determined by MALDI-TOF MS and MS/MS analysis. Proteins were digested and the obtained peptides were separated on a RP column prior the MALDI-TOF MS analysis

| Expected $[M+H]^+$ | Peptide | Measured mass $[M+H]^+$ | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1% soybean [a] | 0.5% soybean 0.5% wheat [a] | 0.25% soybean 0.75% wheat [a] | 2.5 mmol HOCl/g | 5 mmol HOCl/g | Oxidized soybean oil [b] |
| | | | | **Gly m 6 – G1** | | | |
| **979.52** | **$^{357}$Leu-Arg$^{365}$** | **979.52** | **979.42 (39)** | **979.52** | **979.48 (36)** | **979.50 (56)** | **n/d** |
| 1030.50 | $^{121}$Gly-Arg$^{129}$ | 1030.51 (22) | 1030.50 | 1030.53 | 1030.41 (16) | 1030.46 | 1030.42 (16) |
| 1040.58 | $^{36}$Leu-Arg$^{44}$ | 1040.57 | 1040.57 | 1040.57 | 1040.51 (36) | 1040.55 (40) | n/d |
| **1149.55** | **$^{401}$Val-Arg$^{410}$** | **1149.56 (53)** | **1149.43 (59)** | **1149.56 (54)** | **1149.50 (60)** | **1149.53 (66)** | **1149.46 (61)** |
| 1172.60 | $^{482}$Phe-Lys$^{491}$ | 1172.63 | 1172.51 | 1172.62 | 1172.57 (30) | 1172.60 (39) | n/d |
| 1328.68 | $^{482}$Phe-Arg$^{492}$ | 1328.66 | n/d | n/d | 1328.68 (45) | 1328.60 (26) | n/d |
| 1417.65 | $^{195}$Tyr-Lys$^{206}$ | 1417.67 (65) | 1417.65 (73) | 1417.68 | 1417.55 | 1417.51 | n/d |
| **1425.86** | **$^{411}$Val-Arg$^{423}$** | **1425.73 (73)** | **1425.76 (93)** | **1425.74 (72)** | **1425.82 (86)** | **1425.83 (82)** | **n/d** |
| 1742.89 | $^{181}$Arg-Lys$^{194}$ | 1742.93 (53) | 1742.74 (53) | 1742.72 (29) | 1742.81 (8) | n/d | n/d |
| 1978.99 | $^{81}$Arg-Lys$^{97}$ | 1979.00 (32) | 1978.82 (30) | 1979.00 (44) | 1978.92 (96) | n/d | n/d |
| | | | | **Gly m 6 – G2** | | | |
| 1040.58 | $^{33}$Leu-Arg$^{41}$ | 1040.57 | 1040.57 | 1040.57 | 1040.51 (36) | 1040.55 (40) | n/d |
| 1263.65 | $^{228}$Glu-Arg$^{238}$ | 1263.68 (63) | 1263.55 (61) | 1263.53 (66) | n/d | n/d | n/d |
| 1494.69 | $^{192}$Tyr-Lys$^{204}$ | 1494.72 (114) | 1494.70 (102) | 1494.74 (103) | 1494.60 (97) | n/d | 1494.58 |
| 1873.89 | $^{466}$Asn-Arg$^{481}$ | 1873.75 (124) | 1873.82 (130) | 1873.78 (116) | 1873.78 (104) | 1873.88 (132) | n/d |
| 2229.06 | $^{463}$Gln-Arg$^{481}$ | n/d | n/d | n/d | 2029.96 (48) | n/d | n/d |
| 2385.03 | $^{463}$Gln-Arg$^{482}$ | n/d | n/d | n/d | n/d | n/d | 2385.02 (14) |
| 2401.28 | $^{391}$Val-Lys$^{413}$ | n/d | n/d | n/d | n/d | 2401.22 (127) | n/d |
| | | | | **Gly m 6 – G3** | | | |
| 979.54 | $^{343}$Leu-Arg$^{351}$ | 979.52 | 979.42 (39) | 979.52 | 979.48 (40)[§] | 979.50 (56)[§] | n/d |
| 1030.50 | $^{118}$Gly-Arg$^{126}$ | 1030.51 (22) | 1030.50 | 1030.53 | 1030.41 (16) | 1030.46 | 1030.42 (16) |
| 1040.58 | $^{36}$Leu-Arg$^{44}$ | 1040.57 | 1040.57 | 1040.57 | 1040.51 (36) | 1040.55 (40) | n/d |
| 1873.89 | $^{462}$Asn-Arg$^{477}$ | 1873.75 (124) | 1873.82 (130) | 1873.78 (116) | 1873.78 (102) | 1873.88 (130) | n/d |
| 2229.06 | $^{459}$Gln-Arg$^{477}$ | n/d | n/d | n/d | 2229.96 (48) | n/d | n/d |
| | | | | **Gly m 6 – G4** | | | |
| 1192.65 | $^{388}$Leu-Arg$^{397}$ | 1192.66 (26) | 1192.65 | 1192.68 | 1192.58 | n/d | n/d |
| **1398.83** | **$^{408}$Ile-Arg$^{420}$** | **1398.70 (61)** | **1398.74 (64)** | **1398.71 (54)** | **1398.79 (57)** | **1398.80 (30)** | **n/d** |
| 1569.84 | $^{518}$Ala-Arg$^{531}$ | 1569.88 (92) | 1569.71 (58) | 1569.68 (68) | 1569.78 | n/d | n/d |
| | | | | **Gly m 6 – G5** | | | |
| 1398.83 | $^{375}$Ile-Arg$^{387}$ | 1398.68 | 1398.74 | 1398.72 | 1398.79 (60) | 1398.80 (32) | n/d |
| 1543.83 | $^{388}$Gln-Arg$^{400}$ | 1543.66 (55) | 1543.81 | 1543.82 | 1543.75 | 1543.74 | n/d |

*[a] – 48h incubated with 6% glucose at 70°C; [b] – 48h incubated with 1% highly oxidized soybean oil at 70°C; in parenthesis assigned score: >13 = significant homology; > 26 = identity or extensive homology (p<0.05); no parenthesis behind the precursor mass – no MS/MS performed due to lower abundance; n/d – not detected; underlined peptide masses - peptides with one or two missed cleavage; # - Glu ->pyro Glu on the N terminal; §- deamidation (Asn and Gln); shaded – stable peptides*

## 2.4 Development and evaluation of new analytical assays

### 2.4.1 Development of real-time PCR assays for detection of hazelnut and soybean

For the development of new PCR assays for the detection of hazelnut (*Corylus avellana*) and soy (*Glycine max*) we chose to target genes encoding allergens of the respective commodities to ensure a high specificity of the assays. Moreover, amplification of small fragments (50-200 bp) was preferred with regard to detection of the targets in processed food products and to ensure a short analysis time. The genes encoding the allergens *Cor a 1*, *Cor a 8* and *Gly m Bd 28K*, *Gly m Bd 30K* of hazelnut (*Corylus avellana*) and soy (*Glycine max*), respectively, were used as marker genes to design primer pairs and 5'-FAM-labeled, 3'-TAMRA-quenched hydrolysis probes. For each target the available nucleotide sequences were assembled from public databases and aligned to each other using the ClustalW software (Thompson *et al.*, 1997). Following accesion numbers were used for the oligonucleotide design: Z72440 (*Cor a 1*), AF329829 (*Cor a 8*) EU493455, EU493458, EU493457, EU493460, EU493461 (*Gly m Bd 28K*), EU883600, DQ324851 (*Gly m Bd 30K*). Primer and probes were designed on the conserved regions where possible using the Primer 3 tool (Rozen and Skaletsky, 2000) (Table XIII).

Table XIII primer and probe sequences

| Target gene | Designation | Sequence (5'-3') | Amplicon size |
|---|---|---|---|
| *Cor a 1* | Cora1-F | aagatagtggcatcccctca | |
| | Cora1-R | ccagccttaatctgctcgtc | 101 bp |
| | Cora1-P | tgaagagcatcagcaagtaccacaccata | |
| *Cor a 8* | Cora8-F | tgcgtgctctacctgaagaa | |
| | Cora8-R | gtggaggggctgatcttgta | 218 bp |
| | Cora8-P | accgccagtccgcttgcaac | |
| *Gly m Bd28K* | Gly28-F | cgttatctgcagcattgacc | |
| | Gly28-R | cttagccacaagatggcaca | 135 bp |
| | Gly28-P | ccaggtacatgcatgatgcatcca | |
| *Gly m Bd 30 K* | Gly30-F | cacatgcaatagcaacagga | |
| | Gly30-R | tgccatccattgtaacaacc | 94 bp |
| | Gly30-P | ctgaacaagaactcgtagactgtgtggaagaaagc | |

The specificity of the primer pairs for their target within the hazelnut or soybean genome was confirmed by conventional PCR and analysis through agarosegel electrophoresis. This also allowed to check if the amplicon that was formed had the expected length. The selected primers all amplified a single fragment with the expected length. To verify if the designed primer pairs have the potential for primer dimer formation, a melting curve analysis of the PCR products was performed. As the

thermodynamic behaviour of a nucleotide is dependent on its length and sequence, primer dimers will display a different melting profile than the PCR products. Primer dimers can also be detected in the negative control, not containing template DNA. Melting curve analysis resulted in a clear single peak for the different tested primer pairs (Figure 29). Together with the fact that no signal was observed in the negative control, containing no template DNA, it can be concluded that the selected primers do not possess self-complementarity.

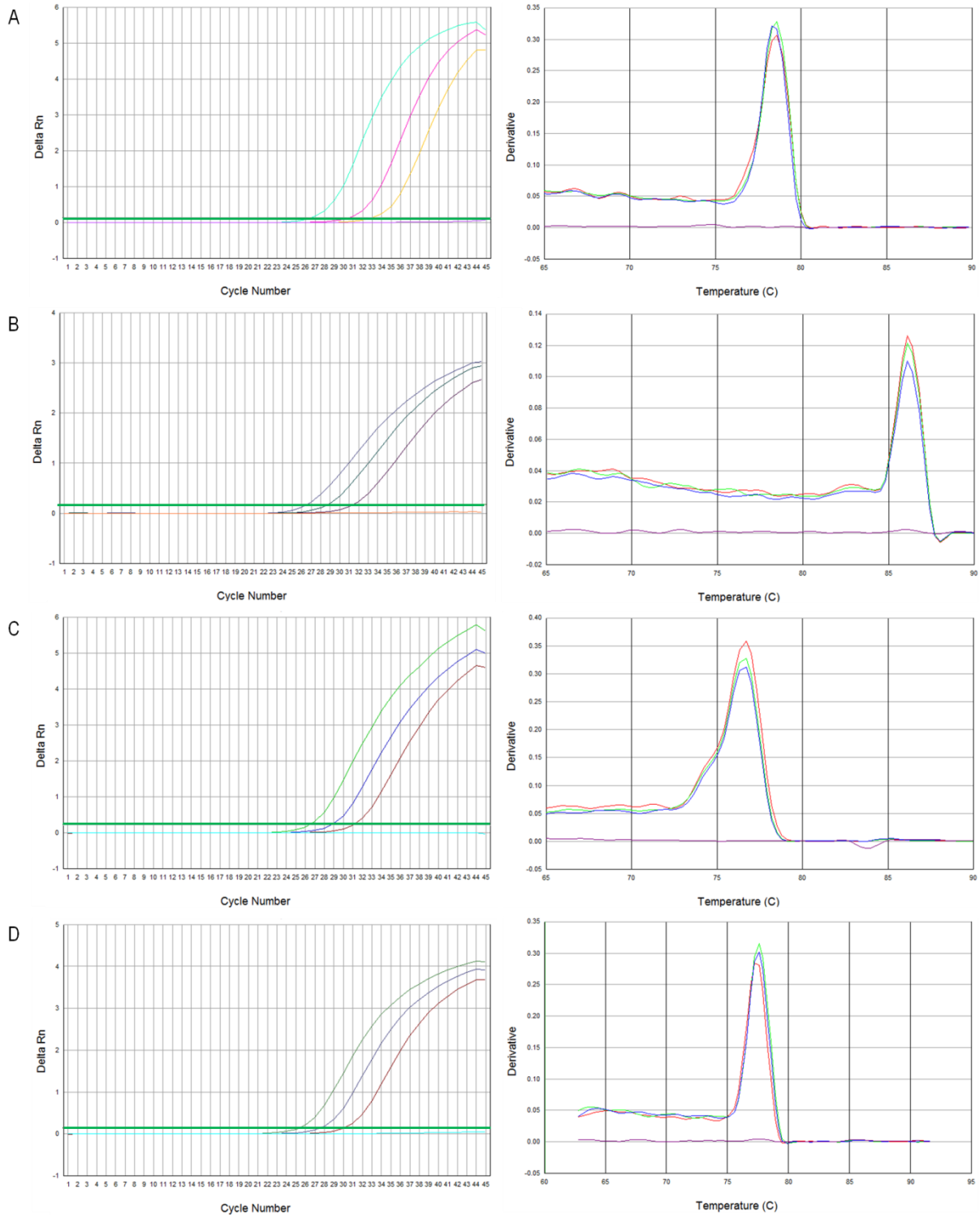


Figure 29 Amplification plots and melting peaks resulting from real time PCR with SYBR Green I detection with the selected primers designed on the gene coding for *Cor a 1* (A), *Cor a 8* (B), *Gly m Bd 28K* (C) and *Gly m Bd 30K* (D)

Successful amplification was achieved with all assays by applying the hydrolysis probes. To achieve the highest level of sensitivity the PCR conditions were optimised. The PCR reactions were performed in a volume of 25 µl containing 2.5 µl (1-10 ng) of template DNA, 1X Real Time PCR Mastermix (Diagenode). Concentrations of 50, 300 and 900 nM forward and reverse primer and 50, 100, 150 and 200 nM probe were used to optimize the PCR conditions. Each run was initiated by a decontamination reaction from dUTP-containing template at 50°C for 2 min, followed by deactivation of the UNG and denaturation for 10 min at 95°C. Each of the 45 cycles consisted of denaturation at 95°C for 15 s and combined primer annealing and elongation at 60°C for 60 s. A combination of 300 nM primers and 200 nM probe yielded the best results in all assays, except for the Gly30-probe, where a concentration of 100 nM resulted in better amplification. These combinations were used in all further runs.

The amplicons of the different assays were sequenced to assess the identity of the products. The fragments which had to be analysed are rather short, in some cases only about 100 bp. The sequencing platform that was used in this study has the internal characteristic to perform poorly on reading the first 50 bp at the 5'-end of the sequence. This means that short fragments of only 100 bp will not be sequenced in an appropriate way and contain a lot of errors. To overcome this a technique described by Binladen *et al*. (2007) was used, who showed that the addition of a 40- or 60-bp nucleotide tail to the sequencing primers enhanced the quality of the sequence obtained from fossil specimens. In this study a tail of 60 bp was used. The approach proved to be successful, resulting in less errors than when the original primers were used in the sequencing PCR reactions. The obtained sequences showed 100 % homology with the sequences from hazelnut and soy, respectively and confirmed the *in silico* determined amplicon sequence of the distinct assays. The PCR products obtained with the commercial PCR assays could not be sequenced as the identity of the primers nor the target is known.

### 2.4.2 Development of a competitive ELISA for hazelnut detection in foods

Several ELISA methods for hazelnut and soybean detection in foods have been reported. However, the ELISA kits, are not always reliable especially when it comes to processed foods because most of the antibodies used for their development are generated against native proteins. Food products are subjected to processing treatments such as heating, pressurization and sterilization which lead to modifications of proteins (Maillard reaction, partial hydrolysis, protein oxidation, etc.). However, when using antibodies towards native proteins, their reactivity towards modified proteins is often decreased as compared to the reactivity towards the native proteins because the specific epitopes are altered during food processing (Cucu *et al.*, 2010b; Downs and Taylor, 2010; Scaravelli *et al.*, 2009). Moreover, the modified

proteins through food processing are often aggregated and their solubility is decreased which renders their extraction from foods difficult. To overcome the issues related to decreases in detectability due to food processing, polyclonal chicken antibodies (IgY) were raised against modified protein extracts. Further, a comparison was made between two approaches to develop antibodies for soybean proteins determination in foodstuffs using polyclonal antibodies developed either towards a single protein such as Kunitz trypsin inhibitor (KTI) or towards modified soybean protein extract.

**Preparation of the immunogen**

The modified hazelnut and soybean extract was obtained by means of their incubation with glucose and sunflower oil . By doing so, the Maillard reaction as well as lipid induced oxidation took place. Hazelnut and soybean protein extracts (20 mg/mL) were incubated for 48 h at 70°C with 120 mg/mL glucose and 10 mg/mL sunflower oil. After incubation, the protein mixture was filtered through a wetted paper filter to remove solid material. Further, precipitation of the proteins was achieved by incubating with TCA 15% (final concentration) on ice for 10 min. After centrifugation (10 min at 10000 g) the obtained supernatant was redissolved in water and the pH of the protein solution was adjusted to 8 using NaOH 5.6 M. A final centrifugation step was performed (10 min at 10000 g) in order to remove any insoluble materials. The protein solution was further stored at -20°C until further use. The modified immunogen was characterized in terms of formation of protein carbonyls, the losses of available lysine residues and changes in the SDS-PAGE pattern as previously described (2.3.3).

**Immunization of chickens**

Polyclonal antibodies were raised in laying hens by injection of the modified extracts and KTI. For the first immunization, 1 mL of 1:1 Freund's complete adjuvant containing 500 µg of modified protein extract were injected intramuscularly. Subsequent injections after 37 days were performed using Freund's incomplete adjuvant containing 500 µg of the same modified protein extract. The eggs were collected starting with the first day of immunization and stored at +4°C until further use.

**Isolation of antibodies from the egg yolk**

The aqueous dilution method was used for the antibodies isolation (De Meulenaer *et al.*, 2005b). Initially, antibodies from one egg each week after first immunization were isolated to assess the antibodies activity. Further, a pool of antibodies, isolated from the eggs collected within the highest activity period, was prepared. The isolated antibodies were stored at -20°C until further use.

### Screening of the antibody's activity using indirect ELISA

The 96 wells microtiter plates were coated (coating buffer: 15 mM $Na_2CO_3$, 35 mM $NaHCO_3$, pH 9.6) with 100 µL of native hazelnut or soybean protein solution (3 µg/mL) and incubated overnight at 4°C. After coating, the unbound material was washed 3 times with washing solution (0.15 M NaCl, 0.05% Tween 20) before 200 µL of blocking buffer (4.5% (w/v) sodium caseinate in phosphate buffer saline (PBS) pH 7.4 which consists of 1.5 mM $KH_2PO_4$, 8 mM $Na_2HPO_4$, 0.135 M NaCl, and 2.7 mM KCl) was added to each well. The plate was further incubated for 1 h at room temperature. The blocking solution was washed 3 times with washing solution before 100 µL isolated antibodies (1:5000 dilution in diluting buffer: PBS pH 7.4, 0.05% Tween 20) were added to each well and incubated for 75 min at 37°C. After a washing step, 100 µL of horseradish peroxidase labeled rabbit anti – IgY IgG solution (1:10000 in diluting buffer) were added and incubated for another 60 min at 37°C. After a final washing step, 100 µL of substrate solution were added to the wells and incubated for 60 min at 37°C. Substrate solution consisted of 0.02% (v/v) TMB, 95.3% (v/v) substrate buffer and 0.007% (v/v) of hydrogen peroxide. The substrate buffer consisted of 15 mM citric acid and 60 mM $CH_3COONa$ with pH 5. Finally, the enzyme reaction was stopped by adding 25 µL of 4 M sulfuric acid to each well. The absorbance was measured at 450 nm using a Bio-Rad Benchmark Plus microplate spectrophotometer (Nazareth, Belgium).

The activity of the antibodies was evaluated on one egg of each week after the first immunization (Figure 30)

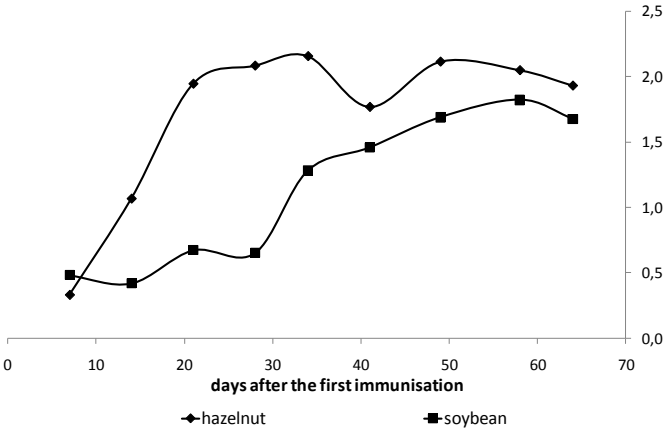


Figure 30 Antibody activity after first immunization

### Indirect competitive ELISA

The indirect competitive ELISA was performed as mentioned above with slight modifications. The plates were coated with 100 µL of 2 µg/mL antigen and incubated over night at 4°C. After the blocking step, 50 µL of sample or standard were added in triplicates together with 50 µL of primary antibodies (1:2000 final dilution) in competition buffer containing 0.3% BSA and 0.6 M NaCl in PBS buffer pH 7.4 and

incubated for 75 min at 37°C. Further, horseradish peroxidase labeled rabbit anti – IgY IgG solution (1:8000 in diluting buffer) were added and incubated for another 60 min at 37°C. All the subsequent steps were performed as previously. Data were evaluated using the SPSS 16 software and calibration curves were fitted using a four-parameter logistic function

$$Abs = \frac{a - b}{1 + \frac{conc}{c}^{d}} + b$$

where abs = absorbance at 450 nm, a = maximum absorbance, b = minimum absorbance, conc = concentration of hazelnut proteins (µg/ml), c – $IC_{50}$ value, concentration at half-maximal saturation (µg/g), d= slope of the curve.

Allergen extraction represents a critical issue when using immunoassays, whose yield depends on both the characteristics of the allergenic protein and the processing to which the allergen containing food was subjected. Chaotropic, disaggregating and reducing agents such as urea, guanidine hydrochloride, SDS and 2-ME are extensively used for the solubilization of proteins. The presence of such agents in the extraction buffers can successfully be used to improve the extractability of the target proteins from the food matrixes (Morishita *et al.*, 2008; Sakai *et al.*, 2010). However, their concentrations should be optimized especially because antibodies used in the ELISA tests can be also affected by the presence of these agents. We have investigated what was the highest concentration of urea to be used in the hazelnut – ELISA and of urea, SDS and 2-ME in the soybean – and KTI - ELISA that was possible to be used without affecting the antigen – antibody interaction. However, concentrations higher than 5 mM urea affected the calibration curves of both the soybean- and hazelnut - ELISA. Moreover, the presence of as little as 0.5 % SDS affected the antigen-antibody interaction in the soybean and KTI - ELISA which was even more pronounced after the addition of the 2-ME. It was therefore decided to use PBS pH 7.4 with 5 mM urea in all further tests as diluting and extraction buffer.

The standards for each ELISA were analyzed at least in 9 replicates. For the hazelnut – ELISA intra-assay variation, the standard deviations and the relative standard deviation (RSD) were calculated based on the results of one plate, while the inter-assay variation was calculated based on the results of 2 different plates. A high repeatability for all the standards was obtained with a coefficient of variation of < 10% for both inter- and intra-assay variance. For the soybean - and KTI- ELISA a coefficient of variation of less than 10% for both inter- and intra-assay variance was obtained for the soybean-ELISA. For the KTI-ELISA intra-assay variance of less than 10 % was also obtained while the inter-assay variance of below 14%.

Further, the detection limit was calculated by taking the mean of at least 20 blanks minus 3 times the standard deviation. A limit of detection (LOD) of 1.4 µg/mL was therefore obtained for hazelnut – ELISA with a limit of quantification (LOQ) of 4.1

µg/mL calculated as 3 times the LOD value. A LOD of 115.6 ng soybean protein/mL was obtained for the soybean - ELISA and of 117.3 ng KTI/mL for the KTI - ELISA. The LOQ value 346.8 ng/mL for soybean – ELISA and 351.9 ng/mL for the KTI - ELISA.

**Cross reactivity studies**

Various nuts, fruits, cereals, legumes and other ingredients were tested. One gram of defatted material was incubated with continuous shaking at room temperature with 10 mL of 5 mM urea in PBS buffer pH 7.4. Afterwards the samples were centrifuged for 20 min at 10000 g and the supernatants were passed through a 0.45 µm cellulose acetate membrane syringe filter. Protein content was assessed via the Kjeldahl method and the extracts were stored at -20°C until further use. When a fitting could be drawn, the cross reactivity was calculating using the $IC_{50}$ value of the hazelnut versus the one of the cross reactant. Otherwise, the equivalent of hazelnut in µg/mL for a certain cross reactant concentration was indicated.

The developed hazelnut - ELISA showed high specificity for the modified hazelnut protein. However, using native hazelnut proteins, the specificity was severely decreased with only 4.3% cross reactivity (Table XIVa). Nevertheless, considering that during processing proteins undergo severe modifications, our purpose was to show that developing ELISA using antibodies raised towards a modified protein extract would be a more reliable approach. Further, the specificity of the ELISA was assessed by testing a number of other tree nuts and food ingredients. In a previous study, Drs et al. (2004) reported that immune-affinity purification of the IgY's is necessary in order to minimize the cross reactivity with other food ingredients. In our case, without any clean up procedures applied, specific IgY could be obtained. It was observed that the maximum cross reactivity was obtained for walnut and pecan nut (8.3 and 5.9 % respectively) while for all other nuts and legumes cross-reactivities of less than 0.6% were obtained (cfr. commercial ELISA kits). For soybean – ELISA high specificity for the modified soybean proteins was observed however relatively low specificity for the native soybean proteins was observed (0.4%). For the KTI-ELISA on the contrary, lower specificity for the modified soybean proteins (5.9%) was observed with a better specificity towards native soybean proteins (43.0%). It is reported that antibodies raised towards soybean proteins might give cross reactivity with other legumes (such as peanut, green pea, lentil, chickpea, beans) due to homology of the proteins. In this case however, insignificant cross-reactivities were observed with these particular foods. Cross-reactivities of below 0.4% were observed with the cacao, pumpkin and macadamia nut proteins in the case of the soybean – ELISA. . For the KTI - ELISA limited cross-reactivity (below 0.55%) was observed with cacao, poppy seed, and sunflower seed proteins. Generally, the developed antibodies had great specificity for the modified soybean proteins, KTI and hazelnut respectively regardless of the fact that no special antibody purification was applied.

Table XIVa Cross reactivity of selected food and food ingredients in the ELISA

| Cross reactant | µg/mL [a] | Soybean - ELISA | | KTI - ELISA | | Hazelnut-ELISA | |
|---|---|---|---|---|---|---|---|
| | | Equivalent (µg/mL) | % cross reactivity | Equivalent (µg/mL) | % cross reactivity | Equivalent (µg/mL) | % cross reactivity |
| Native soybean | 3000 | 13.16 | 0.44 | 645.42 | 43.03 | 0.9 | 0.1 |
| Modified soy | 200 | - | 100 | 11.87 | 2.27[d] | n/a | n/a |
| Coconut | 1640 | 0.83 | 0.05 | 0.16 | 0.01 | 3.6 | 0.2 |
| Macadamia [b] | 1110 | 2.02 | 0.18 | 0.59 | 0.05 | 3.3 | 0.3 |
| Walnut | 180 | n/a | n/a | n/a | n/a | - | 8.2* |
| Pistachio | 7280 | n/a | n/a | n/a | n/a | 0.9 | 0.0 |
| Pine tree | 3070 | n/a | n/a | n/a | n/a | n/d | n/d |
| Almond | 1000 | n/a | n/a | n/a | n/a | n/d | n/d |
| Pecan | 160 | n/a | n/a | n/a | n/a | 9.5 | 5.9 |
| Cashew | 2670 | n/a | n/a | n/a | n/a | 0.2 | 0.0 |
| Brazil nut | 6060 | n/a | n/a | n/a | n/a | 0.3 | 0.0 |
| Red bean | 2170 | 3.68 | 0.17 | 6.28 | 0.29 | n/a | n/a |
| Pumpkin | 1110 | 2.72 | 0.25 | 0.86 | 0.08 | 1.3 | 0.1 |
| Wheat proteins | 3000 | 0.38 | 0.01 | 0.06 | 0.00 | 0.9 | 0.2 |
| Sunflower | 1720 | 2.37 | 0.14 | 2.60 | 0.15 | - | 0.6* |
| Cacao | 1040 | 4.16 | 0.40 | 5.56 | 0.53 | - | 0.6* |
| Native hazelnut | 3000 | 0.00 | 0.00 | 0.14 | 0.00 | - | 4.3* |
| White bean | 6270 | 3.74 | 0.06 | 11.36 | 0.18 | n/a | n/a |
| Poppy seed | 2590 | 2.74 | 0.11 | 5.59 | 0.22 | 4.9 | 0.2 |
| Peanut | 1000 | 0.38 | 0.04 | n/a | n/a | 0.7 | 0.0 |
| Chickpea | 4530 | 2.72 | 0.06 | 1.14 | 0.03 | n/a | n/a |
| Green pea | 4400 | 2.29 | 0.05 | 0.55 | 0.01 | n/a | n/a |
| Sesame | 990 | 1.02 | 0.10 | 0.16 | 0.02 | n/d | n/d |
| Lupine | 9080 | 3.50 | 0.04 | 0.60 | 0.01 | n/a | n/a |
| Modified whey [c] | 2018 | 1.62 | 0.08 | 0.04 | 0.00 | 0.4 | 0.0 |
| Whey | 3000 | 0.39 | 0.01 | 0.06 | 0.00 | n/d | n/d |
| Lentil | 7000 | 3.02 | 0.04 | 2.61 | 0.04 | n/a | n/a |
| OVA | 3000 | 0.12 | 0.00 | n/a | n/a | 0.09 | 0.0 |

[a] – values obtained using the Kjeldahl method; [b] - roasted nuts; [c] – whey modified 48h at 70°C in the presence of glucose and sunflower oil; *- as calculated by dividing the $IC_{50}$ value of the hazelnut by the $IC_{50}$ value of the cross reactant and multiplying by 100, n/d – not detected, n/a – not analyzed

In order to assess the robustness of the developed ELISA, in-house baked cookies were prepared. They were spiked with protein extract at different levels. The impact of the matrix was analyzed by preparing calibration curves in the extract of the blank cookie. The standard curves obtained in the extract of the blank cookies differed significantly from the standard in the diluting solutions as well, indicating therefore matrix effect (Figure 31). To take into account this matrix effect, further experiments were performed by using calibration curves prepared in the respective extracts of the blank cookies.

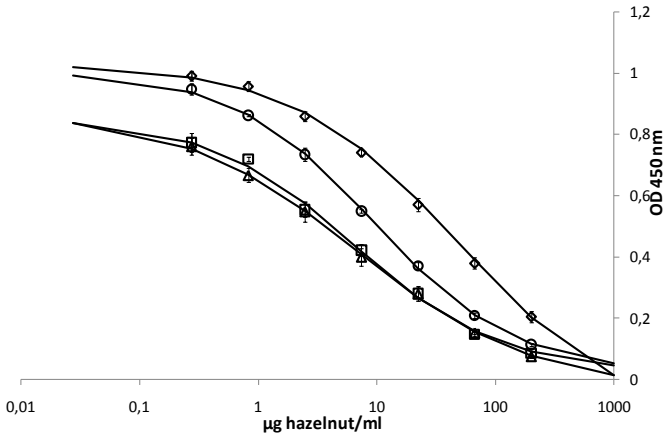


Figure 31 Influence of the matrix on the calibration curve: ◊ - hazelnut protein standards prepared in diluting solution; ○ – hazelnut standards prepared in extract of blank cookie 1 g/10 ml extraction buffer; □ & Δ - hazelnut standards prepared in the extract of the blank cookie 2 g/ 10 ml extraction buffer.

### Recovery studies

Blank and spiked cookies were prepared in-house following AACC method 10-50D (American Association of Cereal Chemists, 2000) as described above (2.2.4, matrix 4). For the cookies spiked with protein extracts, the hazelnut was added to the water phase while for the cookies spiked with defatted powder, the hazelnut was added to the flour. Cookies in which 10% and 15% of the table sugar was replaced by lactose were also prepared and spiked with hazelnut protein extract only. Cookies of 8 cm diameter and about 1 cm thickness were finally baked at 205°C for 10 min. Cookies extracts were further prepared by mixing 2 g of ground material with 10 mL of extraction buffer (5 mM urea in PBS pH 7.4) and incubated for 2 h at 50°C with shaking each 30 min. The samples were further filtered over a whatman filter to remove solid particles and the clear extracts was used directly in the test. Extractions were done in duplicate and each extract was analyzed in triplicate by the indirect competitive ELISA. Further, spiking of the blank cookies before extraction was also performed. After being ground, 2 g of blank cookie was spiked with protein extract at various levels and incubated for either 15 min or 2 h at 50°C with mixing. After incubation the samples were filtered and treated as mentioned above.

When the in-house baked cookies were spiked with hazelnut or soybean proteins before extraction and immediately analyzed by ELISA, high recoveries were obtained for a wider range of spiking levels (Table XIVb). This is in line with the previous studies where high recoveries from the blank foods were obtained as well.

Table XIVb Recovery (%) of the hazelnut and soybean proteins in blank cookies spiked before analysis. Data represent means of minimum 4 independent determinations ± SD

| Spiking level (µg/g) | | Soybean-ELISA | KTI - ELISA | Hazelnut - ELISA |
|---|---|---|---|---|
| Blank cookie | 3 | n/a | n/a | 107.4 ± 23.9 |
| | 10 | 109.0 ± 18.7 | 102.5 ± 19.0 | n/a |
| | 12 | n/a | n/a | 104.8 ± 29.8 |
| | 15 | 114.5 ± 17.1 | 83.2 ± 12.6 | n/a |
| | 25 | n/a | n/a | 82.5 ± 16.7 |
| | 30 | 94.0 ± 8.4 | 90.6 ± 13.5 | n/a |
| | 100 | 104.7 ± 18.6 | 118.0 ± 19.5 | n/a |

*n/a – not analysed*

However, it is well know that food processing is leading to severe protein modifications which (i) can disrupt the antigen-antibody interaction because of the inability of the antibodies to recognize the modified binding epitopes; (ii) thermal processing often leads to reduced solubility of the target proteins lowering the amount of total soluble proteins extracted. Moreover, complexion with food components undergoing thermal processing can induce changes of the physicochemical properties and can sometimes generate protein aggregation hampering as a result the final detection (Monaci and Visconti, 2010). Therefore, the evaluation of the robustness of the ELISA assays using samples spiked before processing is a more correct approach to be applied.

When the cookies were spiked before baking, the recovery of the hazelnut was from 2 to 7% when using PBS with 5 mM urea as extraction buffer (Table XV). No significant differences between spiking with protein extract or defatted hazelnut protein could be seen. The presence of lactose in the recipe, which led to more protein modifications through Maillard reaction, did not additionally affect the recovery of the hazelnut proteins. Unfortunately, in terms of robustness, comparison with other ELISAs developed until now cannot be performed since all the previous studies used spiking before baking.

The recovery of the soybean proteins when spiking cookies before baking is from 3 to 5% for soybean-ELISA and from 10 to 24% when using the KTI-ELISA regardless of the spiking with either soybean protein or defatted powder. The reason for a higher recovery obtained for the KTI–ELISA is probably due to the higher specificity of the antibodies raised against KTI towards the native soybean proteins as compared to the antibodies raised against modified soybean extract. When using 100 % sucrose, which is a non-reducing sugar, limited modifications of proteins are taking place during baking. Considering that the cross – reactivity with the native protein in the soybean – ELISA is low (0.44%) it would have been expected that less than 1% of proteins should be detected. However, proteins seem to be modified

through interaction with the lipids which allowed slightly higher recoveries. For the KTI-ELISA, with a 43% cross reactivity towards the native proteins, lower recoveries are obtained, which are probably due to the above mentioned modifications which hindered the antigen - antibody interaction. Further, the overall lower recoveries obtained when spiking before analysis are obviously also due to the decreased extractability. It is known that the extraction of modified proteins is more troublesome due to aggregation and protein-protein interactions that are taking place. Unfortunately, no clear distinction between these two phenomena can be made from these results. However, in a previous study we have shown that the modification of the proteins alone is already leading to 50 – 100% loss of detectability in commercial ELISA kits for soybean (Platteau *et al.*, 2010a). Moreover, over 50% losses of protein extractability was observed in a model system heated at 120°C for up to 15 min (Figure 32)

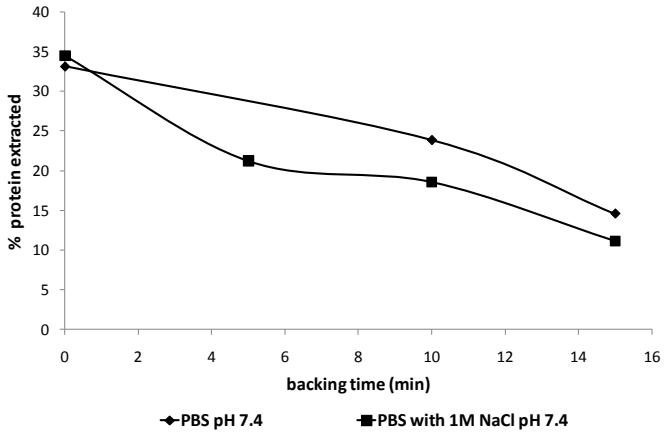


Figure 32 Model system consisting of 50% flour, 20% glucose, 20% sunflower oil and 10% PBS. 10% wheat proteins were replaced with hazelnut protein extract. Model systems (1g) were backed in an oil bath in headspace vials at 120°C. Proteins were extracted by mixing 1g of sample with 10 ml buffer and the protein content was determined by means of Kjeldahl.

When 10 and 15 % lactose were added to the cookies spiked with soybean proteins, which led to more severe protein modifications through Maillard reaction, the recovery in the soybean-ELISA was of 12 to 18 % while in the KTI-ELISA no soybean could be detected anymore. The increased detectability observed in the soybean ELISA is probably due to a better recognition of the more severely modified proteins by the antibodies which were raised against such modified proteins. The detectability using the KTI-ELISA however was completely altered most probably due to the inability of the used antibodies to recognize the severely modified soybean proteins anymore. Nevertheless, the overall recovery is still low, which indicates that the extractability plays a crucial role in food allergen detection.

Table XV Recovery (%) of the hazelnut protein in cookies spiked before backing. Data represent means of 4 independent determinations ± SD

| Spiking (µg/g DM) | | Soybean-ELISA | KTI-ELISA | Hazelnut - ELISA |
|---|---|---|---|---|
| Protein extract | 30 | <LOD | 22.2 ± 2.8 | 7.6 ± 3.6 |
| | 60 | 5.0 ±1.6 | 18.4 ± 2.1 | 4.3 ± 2.1 |
| | 120 | 3.1 ±0.5 | 10.9 ± 2.0 | 3.3 ± 0.9 |
| Defatted powder | 30 | <LOD | 23.8 ± 8.5 | 2.9 ± 0.5 |
| | 60 | 3.9 ± 1.1 | 24.3 ± 7.2 | 2.2 ± 1.0 |
| | 120 | 3.5 ± 1.1 | 10.5 ± 2.1 | 4.2 ± 1.7 |
| Protein extract with 10% lactose | 30 | 18.2 ± 3.5 | <LOD | 2.8 ± 1.3 |
| | 120 | 13.6 ± 2.1 | <LOD | 5.2 ± 0.8 |
| Protein extract with 15% lactose | 30 | 12.6 ± 3.3 | <LOD | 4.7 ± 1.2 |
| | 120 | 12.2 ± 1.6 | <LOD | 4.8 ± 0.8 |

In conclusion, a good selection of the antibodies when developing immunoassays is critical. If using antibodies against one single protein it should be emphasized that the chosen protein should be stable during food processing otherwise false negative results might occur. Even if KTI was shown to be rather stable under thermal denaturation conditions (Roychaudhuri *et al.*, 2003) it seems that using antibodies against KTI alone is not a good strategy since the detectability of soybean proteins is disrupted especially in the processed foods. On the other hand, the use of antibodies against modified protein extracts, would enable to still detect food allergens even in processed foods. Alternatively, a mixture of native and modified proteins can be used for the development of antibodies with similar affinities which will enable to identify modified and non-modified allergens. Moreover, considering the decreased detectability of the hazelnut and soybean proteins in the food products subjected to heat treatment, it is therefore of outmost importance to find possibilities to improve their extractability. Only a good extraction rate from the food products will provide accurate determination of food allergens. It should be therefore emphasized that what is not extracted from the food matrix cannot be detected.

### 2.4.3 Development of a mass-spectrometric method for the detection of hazelnut allergens

#### Detection of hazelnut allergens

The raw hazelnut samples were initially analyzed to determine which allergens are present.

A protein assay (Bio Rad Inc.) was performed on the hazelnut protein extract solution, followed by a trichloroacetic acid, TCA (Sigma, USA)/acetone (Biosolve, The Netherlands)/ DOC (MP Biomedicals) precipitation. Briefly, 1mL sample was mixed with 8 ml 100% ice-cold acetone and then with 1 ml TCA 100%. The proteins precipitated at -20 °C for 1 hr and the sample was centrifuged at 12000 rpm for 15

min at 4 °C. The supernatant was then discarded, and the sample was washed with 1 ml ice-cold acetone, before being centrifuged again at 12000 rpm for 15 min at 4 °C. 150 µg protein was taken for the experiments (200 µg for the home-made cookies). 10 µL 6M guanidium chloride (Sigma, USA), 1% SDS **(**Roche Diagnostics GmbH, Mannheim, Germany)**,** 300mM ammonium bicarbonate (Sigma, USA) was added and the protein concentration was adjusted to 15 mg/mL. Proteins were reduced using dithiothreitol (DTT) (USB Corporation, USA) 10 mM, 56°C, 30 min, and alkylated using iodoacetamide (IAM) (Sigma, USA) 20 mM for 30 min at room temperature RT. More reducing reagent, DTT 11 mM, was then added and was left to incubate at RT for 10 min. The samples were then diluted 30 times with 50 mM ammonium bicarbonate. Sequencing grade bovine trypsin (Roche) was added to the protein mixture at an enzyme-to-substrate ratio of 1:50 (w/w) and was left to incubate for 16 hrs at 37°C. Additional trypsin (1:100 w/w) was added, and the mixture was incubated at 37°C for 3hrs to ensure a complete digestion. Sep paks (Waters, Ireland) were carried out using the following solvents: the phase was activated using 5 mL of the following solvents methanol, MilliQ water, acetonitrile 3x5mL, and MilliQ water. The sample was then loaded into the Sep Paks containing 200 mg of C18 in 3mL volume as packing material. A wash step using 5 mL MilliQ water was performed, followed by the elution steps: a) 15% acetonitrile in MilliQ water, b) then 35%, 50% and 75 % acetonitrile in MilliQ water. The samples were then evaporated to dryness before being resuspended in 125 µL formic acid (Fluka, Sigma Aldrich, Germany) at 1% concentration volume/volume.

The hazelnut or soybean allergens present in the sample were firstly detected, by “bottom up” proteomics using a 2D nanospray ion trap mass spectrometry (Esquire HCT Ultra, Bruker Daltonics, Bremen, Germany).

5 µg/20 µL peptides in 1% formic acid solution was injected. Tryptic peptides were identified by automated data dependent LC-MS/MS. The HPLC is an Ultimate 3000. The peptides were firstly loaded onto a a SCX micro precolumn (500 mm id, 15 mm length, packed with MCA50 bioX-SCX 5 mm; LC Packings) BIOX-SCX (cation exchange column) (5µm-Dionex). The peptides were then passed onto a $C_{18}$ micro-guard column (5µm, 120 Å (Dionex). Four ammonium acetate salt steps were used: 45mM, 75mM, 150mM and 500mM to elute the peptides. analytical column (nano column, reverse phase C18) (75 µm i.d., 15 cm, 3 µm C18 PepMAp100, 300 Å (Dionex). The flow rate for the chromatography was 30nL/min. A multistep linear gradient going from 10 to 40% (0.1% formic acid in 80:20 of acetonitrile/MilliQ water) over 140 min was used. The outlet of the LC system was directly connected to the nanospray source of the ion trap mass spectrometer, controlled by Esquire Control v5.2 and Hystar v3.0 (Bruker Daltonics, Germany). Data acquisition was performed in the mass range of 50–2000 m/z using the standard-enhanced mode (8100 m/z per second). For each MS scan, the 3 most intense doubly or triply charged ions were

selectively isolated and CID was used to induce fragmentation in the trap. Singly charged ions were excluded and dynamic exclusion was used to prevent repetitive selection of the same ions within a preset time.

Smoothing and baseline subtraction were carried out using Data Analysis 3.4. (Bruker Daltonics, Germany). All peaks required a minimum S/N of 6. Data base searching was carried out using BioTools 3.1. (Bruker Daltonics, Germany). The mass tolerances of precursor and fragmented ions were set at 50 ppm and 0.5 Da, respectively. Proteins were identified using the minimally redundant Swiss-Prot *viridiplantae* protein database (release 55.5, SIB; Switzerland), through the MS/MS ion search algorithm of the MASCOT search engine (MASCOT 2.1.04). Only non-redundant peptides with a Mascot score >30 (MS/MS sequencing) were considered in this study. The variable amino acid modifications allowed were oxidation of methionine, carbamidomethylcysteine. One missed cleavage was also allowed.

110 proteins were detected, of which 5 are known allergens for hazelnut.Table XVI provides a list of hazelnut allergens detected by 2D nano LC nanospray ion trap mass spectrometry.

Table XVI List of hazelnut allergens found

| Allergen | Biochemical name | Number of peptides detected | Score | Sequence coverage (%) | Mass (Da) |
|---|---|---|---|---|---|
| Cor a 1 | Pathogenesis-related protein, PR-10 | 3 | 197 | 22 | 17500 |
| Cor a 8 | Lipid transfer protein | 5 | 210 | 49 | 11798 |
| Cor a 9 | 11S globulin-like protein | 20 | 1356 | 41 | 59127 |
| Cor a 10 | Luminal binding protein | 2 | 127 | 4 | 73564 |
| Cor a 11 | 7S vicilin-like protein | 3 | 917 | 32 | 50825 |

**Choice of allergen**

Cor a 9 was one of the five allergens detected and is an 11S globulin-like seed storage protein. Cor a 9 was chosen for the development of the method for the following reasons:

1. It is the most abundant allergen allowing the method to be the most sensitive and is present in all hazelnut varieties. Twenty peptides were identified for Cor a 9 as shown in Table I.
2. Preference was made to choose an allergen susceptible to induce more serious symptoms (Cor a 8, Cor a 9 or Cor a 11), such as life threatening anaphylactic shock, rather than those that induce mild symptoms (Cor a 1 or Cor a 2) generally associated with sensitization to homologues of pollen allergens, predominantly birch (Flinterman *et al.*, 2008).

3. Finally, Beyer *et al*. (2002) previously found that 12/14 patients (86%) had IgE recognizing Cor a 9. Verweij *et al*. (2010) have shown that 60% of infants with atopic dermatitis are sensitized to Cor a 9.
4. Cucu *et al*. (Cucu *et al.*, 2010a) have shown that Cor a 9 is stable during processing (during oxidation and Maillard).

**Choice of peptides**

The following criteria must be fulfilled for the heavy peptide technique. This is explained in more detail by Kirsch *et al* (2007).

1. Must not contain cysteine residues (to avoid an incomplete reduction and digestion of the protein) It is also preferable that no cysteine should be present to avoid an incomplete reduction and digestion of the protein.
2. The chosen peptide must not contain missed cleavages, as otherwise not 100% of the peptide is found in that form and will be detected.
3. Sequence common to all variants of the protein.
4. Peptide specific to a hazelnut allergen to quantify the allergen (or specific to hazelnut to quantify the total amount of hazelnut in the food). A BLAST (Basic Local Alignment Search Tool) was done to determine which peptides are unique to Cor a 9.
5. Easily detectable in MS and MS/MS. A proteotypic peptide (a peptide that fragments well and that gives high peak intensities) must be chosen.
6. Specific fragments produced by MS/MS transitions have to be specific to avoid erroneous quantification of the allergen.

It is preferable to choose a peptide that is not too long to avoid an expensive cost of synthesis. The peptides not fulfilling the first four criteria for the heavy peptide method were eliminated. Table II shows the peptides detected for this allergen (by 2D LC MS/MS analysis of a raw hazelnut extract), and which of these fulfill the heavy peptide criteria.

Six out the twenty peptides shown in Table XVII fulfil the criteria for the heavy peptide method and were chosen to be investigated.

Table XVII Peptides detected for Cor a 9 (by 2D LC MS/MS analysis of raw hazelnut), and which of these fulfill the heavy peptide criteria.

| No. | Score | Peptide | Mr (expt) | Mr (calc) | No Cys | No missed cleavages | Specific | Common to all variants |
|---|---|---|---|---|---|---|---|---|
| 1 | 36 | LQVVRPER | 995.38 | 995.59 | X | X | | |
| 2 | 57 | WLQLSAER | 1001.29 | 1001.53 | X | X | | |
| 3 | 33 | LNALEPTNR | 1026.42 | 1026.55 | X | X | | |
| 4 | 77 | ADIYTEQVGR | 1150.30 | 1150.56 | X | X | | |
| 5 | 43 | YFGECNLDR | 1172.28 | 1172.49 | | | | |
| 6 | 105 | TNDNAQISPLAGR | 1355.42 | 1355.68 | X | X | | |
| 7 | 74 | SRADIYTEQVGR | 1393.4 | 1393.69 | X | | | |
| 8 | 86 | AESEGFEWVAFK | 1398.43 | 1398.65 | X | X | X | X |
| 9 | 96 | INTVNSNTLPVLR | 1439.55 | 1439.81 | X | X | X | X |
| 11 | 84 | DVNGFEETICSLR | 1538.51 | 1538.70 | | | | |
| 12 | 89 | RAESEGFEWVAFK | 1554.49 | 1554.75 | X | | | |
| 13 | 49 | WLQLSAERGDLQR | 1570.52 | 1570.82 | X | | | |
| 14 | 34 | QGQVLTIPQNFAVAK | 1612.61 | 1612.89 | X | X | X | X |
| 15 | 90 | ALPDDVLANAFQISR | 1628.58 | 1628.85 | X | X | X | X |
| 16 | 108 | HFYLAGNPDDEHQR | 1697.47 | 1697.75 | X | X | X | X |
| 17 | 148 | VQVVDDNGNTVFDDELR | 1933.60 | 1933.90 | X | X | X | X |
| 18 | 71 | ADIYTEQVGRINTVNSNTLPVLR | 2571.96 | 2572.36 | X | | | |
| 19 | 51 | GITGVLFPGCPETFEDPQQQSQQGQR | 2902.88 | 2903.35 | | | | |

**Choice of transitions**

A 20 µL peptide mixture was separated using an Alliance 2690 liquid chromatography system (Waters/Micromass, Manchester, U.K.) running a gradient of 10% B to 80% ACN in 11 min 90% H2O containing 0.1% (v/v) acetic acid and ending with 100% ACN over a period of 14 min (total run time 18 min including reconditioning of the LC column) on a Polaris C18 A, 3 µm (2.1mm×150mm) analytical column. 3 µm beads, 200 Å pore size (Varian Inc., Palo Alto, CA, USA). The solvent flow was 400 µl/min. A column heater was used to ensure a stable column temperature of 40°C. A 50 % split of the effluent from the LC column was carried out prior to introduction to the nanospray chamber of the Waters Micromass Quattro Ultima Platinum triple quadrupole mass spectrometer. The triple quadrupole mass spectrometer was operated in positive ion mode. The capillary voltage used was 3 kV, and the source and desolvation temperatures were set at 125 and 250 °C, respectively. The cone and desolvation gas flows were 50 and 650 l/h, respectively. The collision cell pressure was set at $10^{-3}$ bar. The mass spectrometer was used in

daughter scan mode and in MRM mode. The instrument was controlled by MassLynx (v 4.0).

The combination of transitions must be specific to hazelnut. The spectra from each of the peptides satisfying the conditions for Cor a 9 for the heavy peptide technique were compared with respect to specificity of transitions and ion intensities (criteria five and six).

All the possible transitions from the peptides fulfilling the criteria in table II were considered for the heavy peptide method. All the major transitions from AESEGFEWVAFK, INTVNSNTLPVLR, QGQVLTIPQNFAVAK, and HFYLAGNPDDEHQR (peptides n° 8, 9, 14 and 16, respectively, in table II) display many potential interferences (from a variety of food sources, such as rice, chicken, etc.) with similar hydrophobicities to the corresponding peptides from hazelnut and for our chromatography method development have similar retention times, as determined by databases. The best sensitivity and specificity was obtained for ALPDDVLANAFQISR (peptide n° 15 in table II). There are several combinations of transitions that are specific for this peptide and in particular two of the most intense daughter ions ($y8^{+}$ and $y13^{2+}$). The following transitions were chosen: 815.5 → 723.5 Da and 815.5 → 906.5 Da, corresponding to $(M2H)^{2+} \rightarrow y13^{2+}$ and $(M2H)^{2+} \rightarrow y8^{+}$, respectively, from ALPDDVLANAFQISR from raw hazelnut.

The optimal peak intensities for the two transitions, 815.5 → 906.5 Da and 815.5 → 723.5 Da, are obtained at collision energies of 30V and 20V, respectively. The chromatograms for the chosen transitions (at a retention time of 7.4 min) are given in Figure 33. The peptide elutes after 7.4 minutes in the LC gradient.

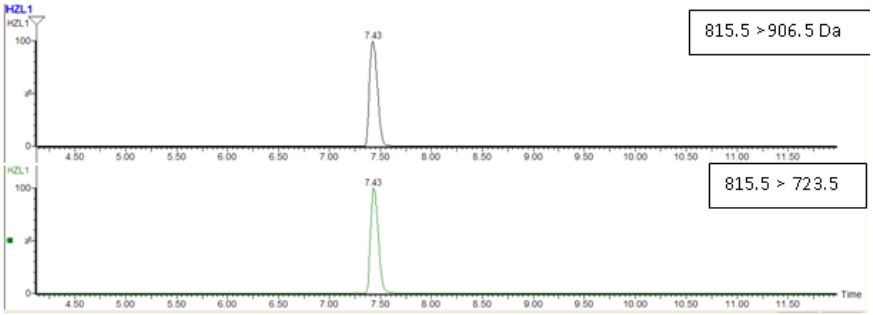


Figure 33 Chromatogram of transitions for ALPDDVLANAFQISR analyzed by LC-MS-MS

The following transitions: 968.0→327.1 Da; 968.0→417.4 Da, corresponding to $(M2H)^{2+} \rightarrow b3^{+}$ and $(M2H)^{2+} \rightarrow y3^{+}$, respectively, at the following collision energies: 30V and 35V from an alternative peptide (VQVVDDNGNTVFDDELR), shown in Figure 34 (with a retention time of 4.4 min).

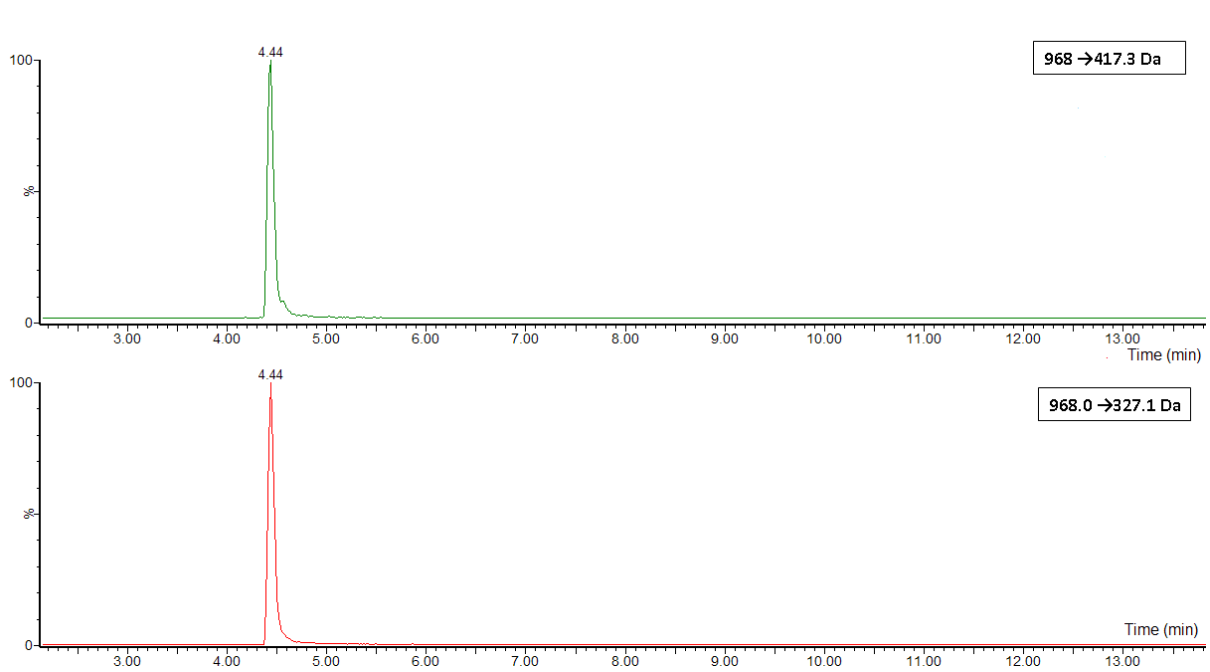


Figure 34 Transitions chosen for VQVVDDNGNTVFDDELR

A disadvantage is the expense of the synthesis of the heavy peptide, due to the length of the peptide for a quantitative method.

The mass tolerance for the parent and fragment masses is **±** 0.5 m/z, and for complex matrices, especially, interfering transitions could arise. If this is the case, this alternative peptide can be used. The use of the two peptides will be compared for a cookie sample later on.

**Analysis of food processing imitation samples**

Model food systems were prepared by baking (at 120°C) a mixture of 50% flour (Carrefour own brand/Belgium), 20 % sunflower oil (Vandemoortele), 20 % sugar (Béghuin Say) and 10 % PBS (pH 7.4). 10 and 20 % of the wheat protein was substituted with 10 and 20 % hazelnut, heated for 0, 5, 10 and 15 min, as shown in table VI. Protein extraction and sample preparation was carried out as described in the material and methods section.

The following ingredients were mixed together to make the home-made cookies: butter (Carrefour own brand) 64.0 g; sugar (Béghuin Say) 131.8 g; salt (Cérébos) 2.1 g; sodium bicarbonate (Cérébos) 2.5 g; water 49.3 g; flour (Carrefour own brand) 225.8 g. The dough was spiked such that the hazelnut protein extract was 160 ppm. A second batch of cookies with the same recipe was made, this time spiked such that it had 100ppm hazelnut protein extract in the dough. For soy the dough was spiked with 0, 50 (50 µg/g), 500 and 2500 ppm soybean protein extract/g dough.

Following extraction protocol was used for the food samples. 1g of each shop-bought chocolate (Carrefour), home-made or shop-bought cookies (Lu, Kraft) was weighed. Cookies were ground with a blender. Chocolate samples were ground with

a blender then melted using a water bath. 10 ml of hexane (Carlo Erba Reactifs-SDS, Val de Reuil, France) per gram of food were used to extract lipids. The defatting step was repeated 3 times. To extract proteins, 3ml of 8M urea (Sigma-Aldrich Chemie GmbH) in Tris (Invitrogen, Paisley, Scotland, UK) buffer pH 8 was added and incubated 1h at RT. This step was repeated twice, the last step being incubated overnight at RT. Samples were then centrifuged at 2500 rcf (relative centrifugal force). The clear supernatant was used.

Table XVIII and Table XIX show the conditions used for the food processing imitation reactions for hazelnut samples. For the first set of samples (Table XVIII), different concentrations of hazelnut proteins were heated (at 70°C) in the presence of wheat or casein proteins, and/or glucose for different incubation times.

Table XVIII Conditions used for food processing imitation reactions for hazelnut samples (Temperature = 70°C)

| Model system | Incubation time (h) | Hazelnut (mg/mL) | Wheat (mg/mL) | Casein (mg/mL) | Glucose (mg/mL) |
|---|---|---|---|---|---|
| 1; 2; 3; 4 | 0; 21; 30; 48 | 2.5 | 7.5 | | 60 |
| 5 | 0; 21; 30; 48 | 5 | 5 | | 60 |
| 9 | 0 ; 48 | - | - | 10 | 60 |
| 11 | 48 | - | - | 10 | - |
| 12 | 0 ; 48 | 0.1 | - | 9.9 | 60 |
| 14 | 48 | 0.1 | - | 9.9 | - |
| 15 | 0 ; 48 | 2 | - | 8 | 60 |
| 17 | 48 | 2 | - | 8 | - |
| 18 | 0 ; 48 | 4 | - | 6 | 60 |
| 20 | 48 | 4 | - | 6 | - |
| 21 | 0 ; 48 | 8 | - | 2 | 60 |
| 23 | 48 | 8 | - | 2 | |

Table XIX Model systems containing hazelnut

| Sample | Incubation time (min) | % hazelnut | % wheat |
|---|---|---|---|
| 24 | 0 | 10 | 90 |
| 25 | 5 | 10 | 90 |
| 26 | 10 | 10 | 90 |
| 27 | 15 | 10 | 90 |
| 28 | 0 | 20 | 80 |
| 29 | 5 | 20 | 80 |
| 30 | 10 | 20 | 80 |
| 31 | 15 | 20 | 80 |

For the second set of samples (Table XIX), different concentrations of hazelnut proteins were heated (at 120°C) in the presence of wheat proteins, for different incubation times.

All the 31 samples of tables 3 and 4 were firstly analyzed “bottom up”, by 2D nano LC nanoESI IT, to determine which hazelnut peptides were present. Three samples in table III are negative controls (samples n° 9, 10, 11), not containing any hazelnut, and indeed no hazelnut peptides were detected. Cor a 9 was detected in all samples (except the negative controls). Extreme conditions have been tested, such as heating for 48 hours at 70°C**.**

The peptides ALPDDVLANAFQISR and VQVVDDNGNTVFDDELR were detected in all the food processing imitation samples from Table XVIII and Table XIX, even after heating for up 15 min at 120°C in the presence of other compounds, including glucose (apart from the negative controls in table III). The only allergen present in all the food processing imitation samples was the 11S globulin-like protein (Cor a 9), the protein chosen for the detection method for raw hazelnut, confirming the fact that Cor a 9 is the most abundant and the best choice for the target peptide. This choice was also confirmed by the literature. It has previously been shown that Cor a 1.04 (homologous to Bet v 1, the major birch pollen allergen) (Gerber *et al.*, 2003) and Cor a 8 (lipid-transfer protein) are also the dominating hazelnut allergens. They were less abundant than Cor a 9 and were not detected in all samples after food processing (Lauer *et al.*, 2004; Ortolani *et al.*, 2000). It has previously been shown that heat treatment substantially reduces the reactivity of Cor a 1 (Muller *et al.*, 2000; Schocker *et al.*, 2000; Worm *et al.*, 2009). Roasting of hazelnuts has been shown to significantly decrease the allergenicity of the nuts (Hansen *et al.*, 2003).

**Optimisation: comparison of the use of the two selected peptides**

Table XX shows a comparison of peak intensities for the methods optimized for VQVVDDNGNTVFDDELR and ALPDDVLANAFQISR for an industrial cookie.

Table XX Comparison of peak intensities for the methods optimized for ALPDDVLANAFQISR and VQVVDDNGNTVFDDELR

| Transitions for ALPDDVLANAFQISR | Peak intensities | Transitions for VQVVDDNGNTVFDDELR | Peak intensities |
|---|---|---|---|
| 815.5>723.5 Da | 121844.7 | 968.0>417.4 | 6684.34 |
| 815.5>906.5 Da | 17889.41 | 968.0>327.2 | 39077.56 |

The method using transitions from ALPDDVLANAFQISR is clearly more sensitive than that with VQVVDDNGNTVFDDELR, as the peak intensities for the transitions for the first peptide are more intense than those for the second. Both methods have the correct ion ratios, and clearly work for the industrial cookie samples analyzed. If

there are foods where there are interferences for ALPDDVLANAFQISR (either the blanks show interfering peaks, or the ion ratios are not correct), the second method with VQVVDDNGNTVFDDELR can be used instead.

There are not yet guidelines for mass spectrometry analysis of proteins, and therefore we refer to guidelines for veterinary drug residue analysis. According to the European Commission decision 2002/657/EC, if the relative intensity (% of base peak) of each peak with respect to the base peak is between 10 and 20 % of the base peak, the allowed error variability on the other peaks is 30 %.

**Analysis of home-made cookies spiked at 160 ppm**

Three samples from three different cookies from a batch made with hazelnut protein extract were analyzed. The correct ion ratios were obtained for the cookies (between 5.4 and 10.1). As a good signal was obtained for these cookies, the quantity of hazelnut in the next batch of cookies will be reduced to 100 ppm. An internal standard will be injected with the next lot of samples to enable quantification of Cor a 9.

### 2.4.4 Development of a mass-spectrometric method for quantification of hazelnut allergens

**Heavy peptide transitions**

With the heavy peptide method, the same (peptide n° 15 in Table XVII) peptide, labelled isotopically is used as an internal standard. The peptide: ALPDDVLANAF(10)QISR was synthesized (Heavy Peptide$^{TM}$ AQUA, 2 vials x 2nmol, Thermo Scientific, Belgium). The samples for home-made cookies, shop-bought cookies, and chocolate were firstly analyzed by LC-MS-MS without addition of the heavy peptide to ensure that there was no interference at these masses and retention time. The transitions given in Table XXI for the heavy peptide were chosen, as there was no interference at the required retention time. Two transitions for the heavy peptide were required in order to determine that there is no interference for the heavy peptide and that the quantification is correct.

Table XXI Transitions from ALPDDVLANAFQISR from Cor a 9 (11S globulin-like protein) and the heavy labelled peptide chosen

| | Transition | Fragment ion type | Collision energy (V) |
|---|---|---|---|
| ALPDDVLANAFQISR | 815.5 → 723.5 Da | $y13^{2+}$ | 20 |
| | 815.5 → 906.5 Da | $y7^{+}$ | 30 |
| ALPDDVLANAF(10)QISR | 820.9 →728.5 Da | $y13^{2+}$ | 20 |
| | 820.9 →611.4 Da | $b6^{+}$ | 25 |

Five different collision energies (15, 20, 25, 30 and 35 V) were tested for the heavy peptide. One transition (815.5 > 723.5) was the same for the heavy peptide as for the naturally occurring peptide (a mass difference of 5 Da due to the fact that the ion is doubly charged), and the optimal collision energies were of course the same (as shown in Table XXI) for this transition. However, for the second transition for the naturally occurring peptide, there was some interference at the required retention time, and a different transition was chosen. The chosen heavy peptide transitions are shown in Figure 35 (although the collision energy is not optimal for the fragmentation to obtain the second heavy peptide fragment).

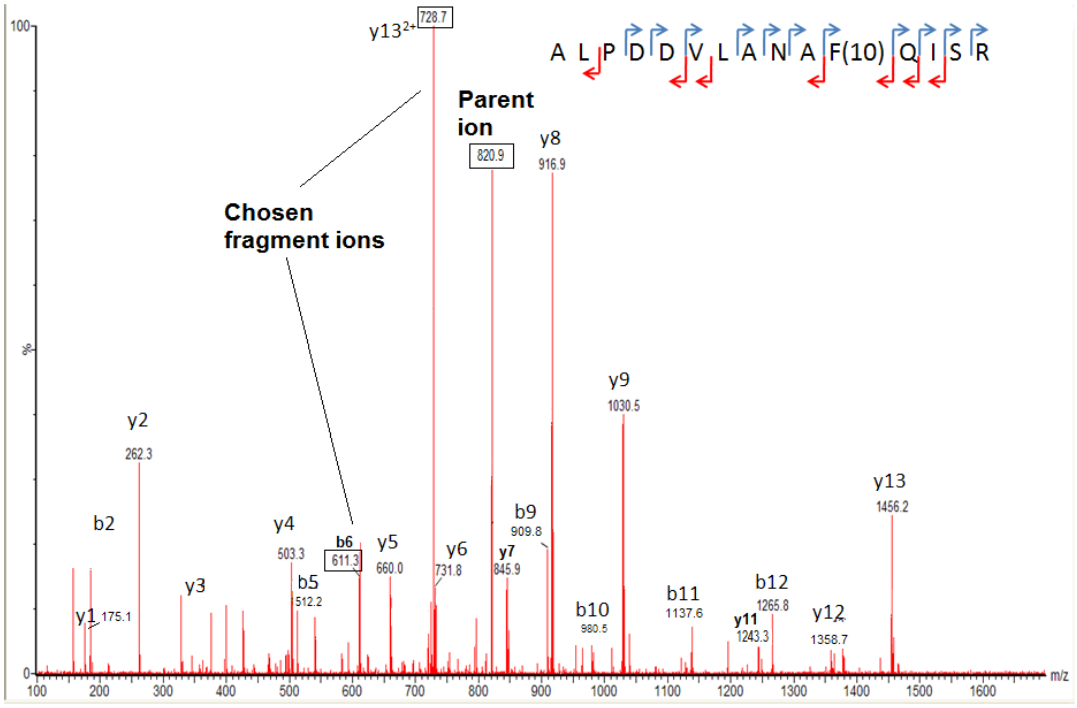


Figure 35 Heavy peptide transitions ALPDDVLANAF(10)QISR)

### Quantification with real samples

The method was tested on three different shop-bought cookies and chocolate (plain chocolate with a truffle containing hazelnut in the middle, and on home-made cookies, spiked with 100 ppm hazelnut protein extract before baking. Table XXII shows the quantification results for Cor a 9 in each. The quantities of Cor a 9 in each sample were determined from the quantification of the target peptide using isotopic dilution with a heavy isotopically labelled peptide (internal standard: ALPDDVLANAF(10)QISR).

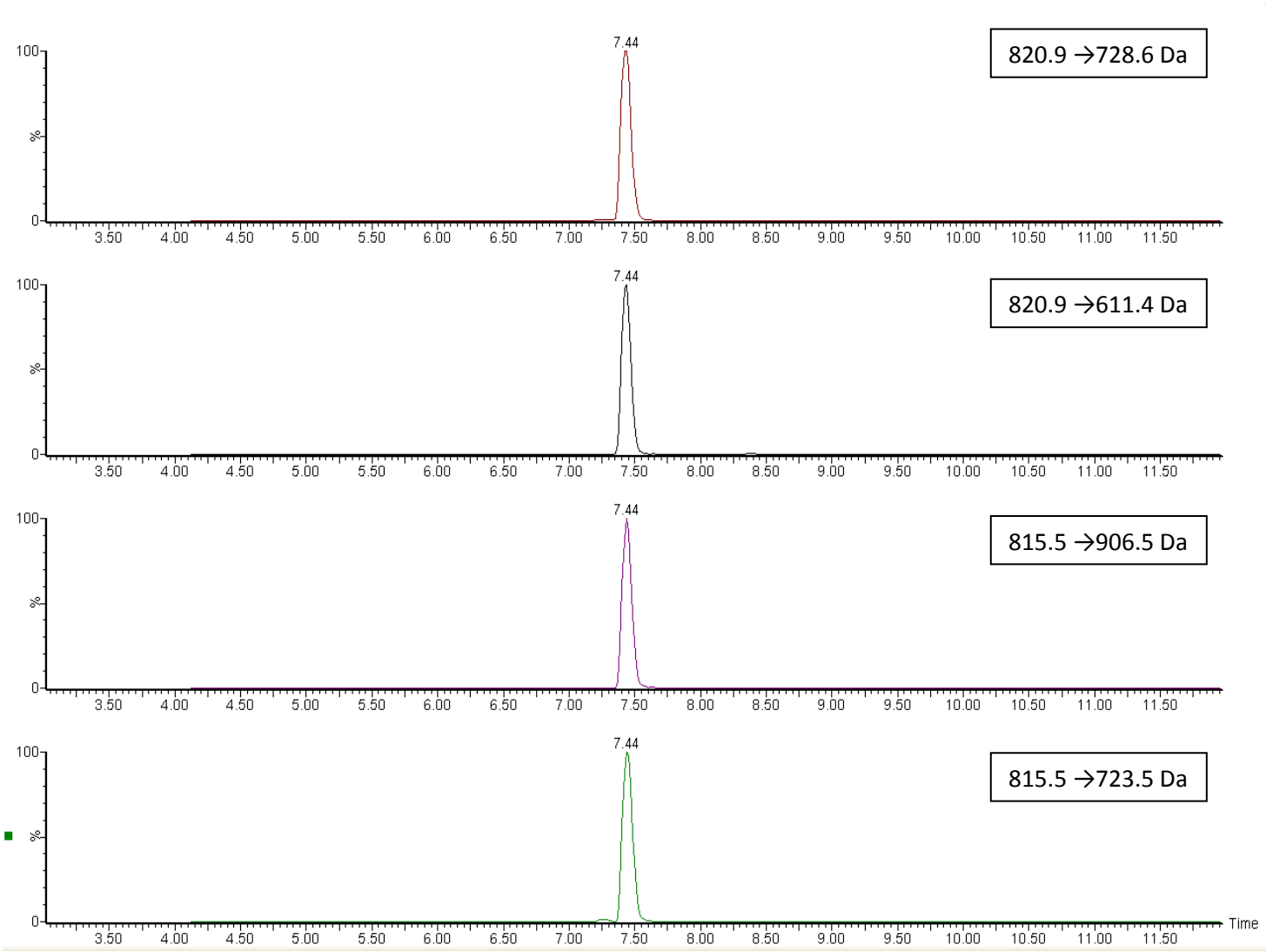


Figure 36 Chromatogram of the heavy peptide transitions (LC-MS/MS analysis: injection of a known amount of heavy peptide)

Table XXII mg (ALPDDVLANAFQISR and Cor a 9) /kg food: ppm

| Peptide/ allergen | Shop-bought chocolate | Home-made cookies | Shop-bought cookies |
|---|---|---|---|
| ALPDDVLANAFQISR | 7.30±0.51 | 0.19±0.01 | 3.68±0.49 |
| Cor a 9 | 185.59±18.33 | 6.85±0.52 | 133.49±17.96 |

The method is sensitive for the detection of the peptide ALPDDVLANAFQISR from Cor a 9. For the home-made cookies spiked with 100 ppm of hazelnut protein extract (corresponding to 19.95 ppm Cor a 9 for this mixture of hazelnut varieties), as low as 0.17 ppm ALPDDVLANAFQISR was detected, corresponding to 6.32 ppm of total allergen (taking into account the masses for the peptide and for the allergen). The final amount of hazelnut protein extract spiked must increase slightly after baking due to evaporation of water. The LOQ has not been reached and remains to be

determined. Validation will be necessary to determine the LOD and LOQ. The sensitivity of the method is comparable to that obtained by PCR and ELISA methods for the detection of hazelnut (<10ppm) (Akkerdaas *et al.*, 2004; Bettazzi *et al.*, 2008; D'Andrea *et al.*, 2009; Holzhauser and Vieths, 1999; Schoringhumer *et al.*, 2009).

The aim is to develop a method to protect allergic consumers, and so the method needs to be as sensitive as possible. The peptide does not give any information as to the allergenicity of the protein, however, if the peptide is detected, then the allergen is present.

The ion ratios for the two transitions for all the home made cookie and the shop-bought chocolate samples were well within the limits of acceptability. The ion ratios for the shop bought cookie samples were all within the limits of acceptability, however, as there was some variation in the ion ratios, then when the standard deviation of the ion ratios is calculated (6.26 ± 1.02) and taken into account, 6.26-1.02=5.24, this value is outside the permitted range of between 5.4 and 10.1. As this work will be validated using many more samples, as compared to those obtained by the ELISA method, it could be that different transitions may be required.

There is around twenty seven times more Cor a 9 in the chocolate than in the home-made cookies, and around 10 times more in the industrial cookies than the home-made ones, spiked with 100 ppm hazelnut protein. This is to be expected due to the higher quantities of hazelnut present, as the industrial cookies and chocolate were chosen to test that the method works, containing small quantities of hazelnut. For the industrial cookies, Table XXI shows that one cookie part contained 113.70 ppm of Cor a 9, instead of the 148.69 and 138.11 ppm in the other two cookies. This is due to the presence of different amounts of nougat in the different parts of the cookies (it is not that homogeneous). The cookie parts are from the same packet, however, from different cookies. The quantity of allergen in the shop-bought cookies and chocolate depends on the hazelnut variety used. There are surely differing amounts of Cor a 9 in the different hazelnuts used in each product.

### 2.4.5 Development of a mass-spectrometric method for the detection of soybean allergens

#### Soybean allergens detected (nanoLC ESI IT)

The soybean allergens present in the sample were firstly detected using a 2D nano LC ESI IT, as described above for hazelnut allergens. Fifty four soybean proteins were detected, of which there are five known allergens: P34, β conglycinin, glycinin**,** lectin agglutinin, Kunitz trypsin inhibitor (listed in Table XXIII).

Table XXIII Allergens detected in raw soybean (list not including hull proteins)

| Allergen | Biochemical name | Number of peptides detected | Mass (Da) | Score | Sequence coverage (%) |
|---|---|---|---|---|---|
| Gly m 1 (Gly m Bd 30K) | P34 | 2 | | | |
| Gly m 5.0101 | β conglycinin α subunit | 14 | 63127 | 401 | |
| Gly m 5.0201 | β conglycinin α' subunit | 10 | 65103 | 345 | 15 |
| Gly m 5.0301 | β conglycinin β subunit | 8 | 48301 | 521 | 22 |
| Gly m 6.0101 | glycinin G1 | 6 | 55672 | 423 | 18 |
| Gly m 6.0201 | G2 | 6 | 54357 | 404 | 18 |
| Gly m 6.0401 | G4 | 8 | 63641 | 668 | 40 |
| Gly m lectin | lectin, agglutinin | 2 | | | |
| Gly m TI | Kunitz trypsin inhibitor | 7 | 23990 | 420 | 31 |

**Choice of allergen**

The allergens β conglycinin (7S storage protein) (Frias *et al.*, 2008; Ogawa *et al.*, 2000; Velasquez, 2007) and glycinin (11S globulin) were chosen because:

1. Most peptides were detected for the storage proteins β-conglycinin (32 peptides) and glycinin (24 peptides). As the proteins are large, there are several possibilities for a choice of peptide. It has been reported that the most abundant protein in soybean is either glycinin or β conglycinin, depending on the variety, genotypes, maturity, etc. (Derbyshire, 1976). It is important to work on the most abundant allergens, to enable maximum sensitivity of the method.
2. P34 is the immunodominant allergen (Babiker *et al.*, 1998; Helm *et al.*, 1998; Maruyama *et al.*, 2003; Wilson *et al.*, 2005; Yaklich *et al.*, 1999), however, this protein is known to be found in concentrations less than 1% (Wilson *et al.*, 2005). The LOD and LOQ would be reached more quickly if a peptide from this protein were chosen. It has previously been shown that 80% of Japanese soybean varieties do not contain Gly m Bd 28K (Bando, 1996). False positives may be observed if the soy does not contain the allergen.

It has previously been shown that about 25% of soybean-sensitive Japanese patients with atopic dermatitis were allergic to the α-subunit of *β*-conglycinin (Krishnan *et al.*, 2009), which is therefore a major allergen. It has previously been

found that the allergenicity of the β conglycinin (7S globulin) is higher than that of the glycinin (11S globulin) (Keum *et al.*, 2006; Lee *et al.*, 2007). More peptides were detected for β conglycinin (32 peptides), indicating that it may be found in slightly higher concentrations in this soy variety than glycinin (24 peptides were detected). In any case, both proteins are found in all soy varieties.

**Choice of peptides**

The following criteria must be fulfilled for the SRM technique. This is explained in more detail by Kirsch *et al.* (2007).

7. The sequence must be common to all variants of the protein, otherwise false negative results may be observed.
8. The peptide must be specific to a soy allergen to quantify the allergen (or specific to soy to quantify the total amount of soy in the food). A BLAST (Basic Local Alignment Search Tool) was done to determine which peptides are unique to glycinin and β conglycinin, otherwise false positive results could be observed if the peptide, present in another species is detected. It is very important that the transitions should be specific to soy, and that no interfering transitions from any species found in even trace amounts in food, be present.
9. The peptide should not contain cysteine residues (to avoid an incomplete reduction and digestion of the protein).
10. The chosen peptide must not contain missed cleavages, as otherwise not 100% of the peptide is found in that form and will be detected.
11. Chosen fragments produced by MS/MS have to be specific to avoid erroneous identification of the allergen.
12. The peptide should be easily detectable in MS and MS/MS. A proteotypic peptide (a peptide that fragments well and that gives high peak intensities) must be chosen.
13. It is preferable to choose a peptide that is not too long to avoid an expensive cost of synthesis.

The peptides not fulfilling the first two criteria for the SRM method are firstly eliminated.

**Comparison of the chosen transitions from the peptides from β conglycinin α chain and glycinin G1**

Table XXIV and Table XXV show the peptides detected for these allergens (by 2D LC MS/MS analysis of a raw soybean extract), and which of these fulfill the criteria discussed above. Table XXIV shows peptides satisfying the conditions discussed above (for SRM) for β conglycinin (α chain) and which have been eliminated. Sixteen peptides were detected, none of which contain cysteine residues and eleven of these have no missed cleavages. Eleven peptides are sequences common to all variants, however, and only three of these are specific to soy (peptide specificity is determined

with the protein NCBI BLAST program. Only three peptides from the sixteen originally detected are therefore potential candidates for the SRM method.

Table XXIV Peptides satisfying the conditions for the heavy peptide for β conglycinin (α chain)

| Mr (expt) | Score | Peptide | No Cys | No missed cleavages | Sequence common to all variants | Specific |
|---|---|---|---|---|---|---|
| 954.51 | 37 | R.SPQLQNLR.D | X | X | X | |
| 1009.53 | 45 | K.FFEITPEK.N | X | X | X | |
| 1050.59 | 40 | **K.NPFLFGSNR.F** | X | X | X | X |
| 1152.57 | 41 | R.NILEASYDTK.F | X | X | X | |
| 1182.53 | 60 | **R.ESYFVDAQPK.K** | X | X | X | X |
| 1390.89 | 63 | **R.LITLAIPVNKPGR.F** | X | X | X | X |
| 1405.73 | 55 | K.TISSEDKPFNLR.S | X | X | X | |
| 1438.66 | 52 | R.ESYFVDAQPKKK.E | X | | | |
| 1533.80 | 44 | R.KTISSEDKPFNLR.S | X | | | |
| 1617.77 | 58 | K.FFEITPEKNPQLR.D | X | | | |
| 1938.68 | 100 | K.GSEEEDEDEDEEQDER.Q | X | X | X | |
| 2024.93 | 59 | K.EQQQEQQQEEQPLEVR.K | X | X | X | |
| 2037.84 | 49 | R.QEEEHEQREEQEWPR.K | X | | | |
| 2151.06 | 114 | R.VPSGTTYYVVNPDNNENLR.L | X | X | X | |

Make sure add up to correct score and no. of peptides

Only three of the ten peptides detected from glycinin G1 fulfill criteria 1 to 7, discussed above, as shown in Table XXV. Most peptides were eliminated from the BLAST results, as the same peptides were found in other species, and are therefore not specific to soy. Indeed, glycinin has homologues in other species, such as hazelnut and peanut. The target peptide must be unique to the protein of interest. If this is not the case, the protein of interest might be overestimated and this can lead to false positives, as discussed by Sherman *et al*. (2009) and Duncan *et al*. (2009)

Table XXV Peptides satisfying the conditions for the heavy peptide for glycinin G1

| **Mr(expt)** | **Score** | **Peptide** | No Cys | No missed cleavages | Sequence common to all variants | Specific to soy |
|---|---|---|---|---|---|---|
| 978.51 | 62 | **R.LSAEFGSLR.K** | X | X | X | X |
| 1148.60 | 77 | **R.VFDGELQEGR.V** | X | X | X | X |
| 1171.60 | 16 | **K.FLVPPQESQK.R** | X | **X** | X | X |
| 1371.63 | 54 | R.ALIQVVNCNGER.V | | | | |
| 1424.92 | 63 | R.VLIVPQNFVVAAR.S | X | X | X | |
| 1449.64 | 74 | R.SQSDNFEYVSFK.T | X | X | X | |
| 1585.73 | 68 | R.FYLAGNQEQEFLK.Y | X | X | X | |
| 1741.85 | 107 | R.RFYLAGNQEQEFLK.Y | X | | | |
| 1899.92 | 68 | K.NLQGENEGEDKGAIVTVK.G | X | | | |
| 1977.92 | 71 | R.RPSYTNGPQEIYIQQGK.G | X | X | X | |

### Choice of transitions

The peptides were analysed as described above for the hazelnut peptides. The spectra for each of the peptides, fulfilling the criteria discussed in part 4.3, from these two soybean allergens were compared with respect to the specificities of the transitions and the peak intensities.

From the three peptides from β conglycinin, the peptide NPFLFGSNR was chosen. The following two transitions (parent ion into fragment ion) from this peptide are specific to soy: m/z 526.3 → 580.1 Da (y5) and m/z 526.3 → 840.5 Da (y7), corresponding to $(M+2H)^{2+}$ → y5 and $(M+2H)^{2+}$ → y7, at a collision energy of 15 V for all. The daughter scan MS/MS spectrum for this peptide is given in Figure 37. However, the peak intensities were lower for this peptide than for VFDGELQEGR, from glycinin, when injected at the same concentration. The following transitions (Figure 38) are sufficiently specific for the detection of soy in all the samples planned for this work: 575.31→903.417 Da; 575.31→788.390 Da, corresponding to $(M+2H)^{2+}$ → y8 and $(M+2H)^{2+}$ → y7 at the optimal collision energy of 15 and 10 V, respectively.

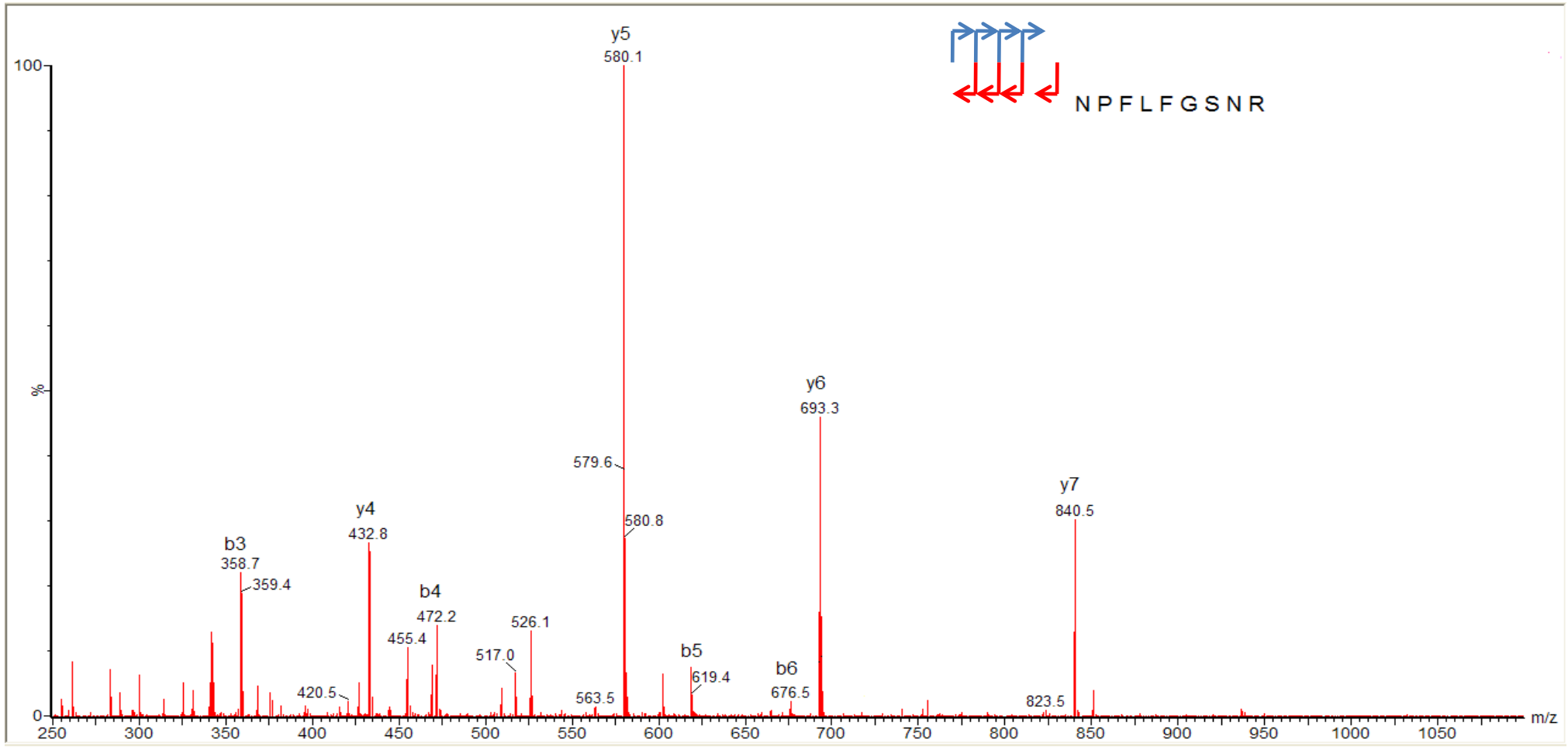


Figure 37 Daughter scan MS/MS spectrum for VFDGELQEGR (glycinin G1)

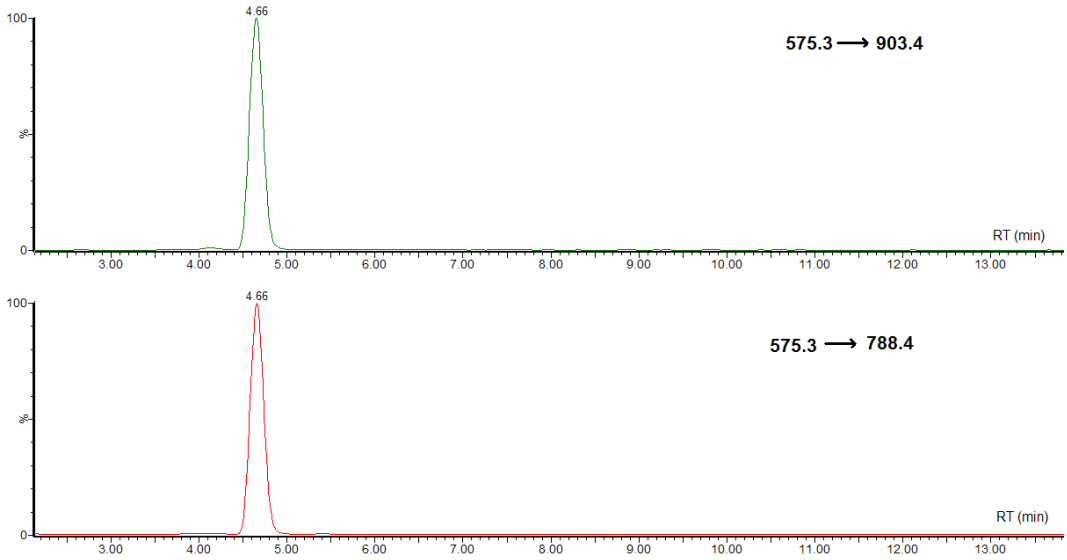


Figure 38 Chromatograms for the two chosen transitions for VFDGELQEGR

The chosen transitions were not detected in blank cookies (there are therefore no interfering peaks from the cookie matrix), as shown in Figure 39.

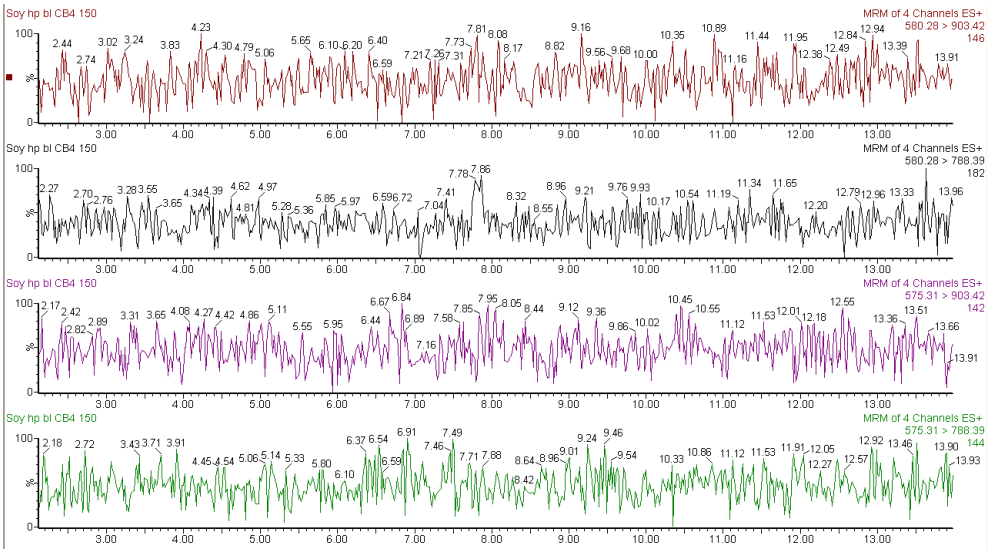


Figure 39 Chosen transitions in blank cookies.

Figure 40 shows the daughter scan MS/MS spectrum for VFDGELQEGR (glycinin G1), at a collision energy of 15V. There is an intense fragment peak, and several less intense fragment ion peaks for this peptide for the choice of the transitions.

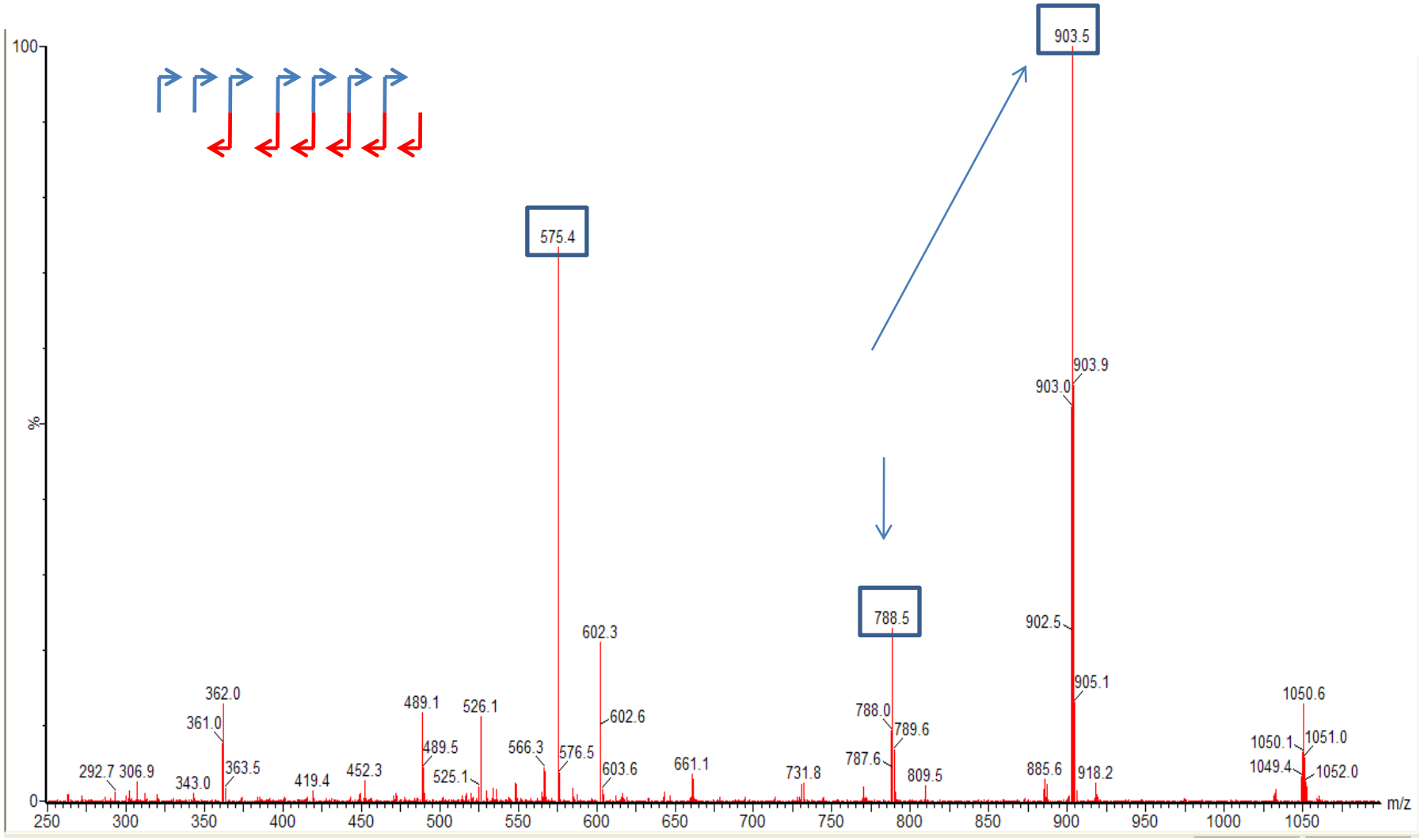


Figure 40 Daughter scan MS/MS spectrum for VFDGELQEGR (glycinin G1)

### Analysis of food processing imitation samples

Sixteen soy food processing imitation samples were analysed (shown in Table XXVI) to determine if these peptides were still the most adapted, in order to choose a peptide, which at least partially resists heat treatment and the reactions taking place during baking. A range of factors were investigated, including temperature, incubation time, presence of glucose, and of other proteins from wheat and casein. Samples in groups A, B and C were incubated at a temperature of 70 °C. The samples in groups D and E were model cookie systems and were heated to 120 °C.

Table XXVI Maillard reaction soy samples

| Model system | Incubation time (hrs) | Soy (mg/mL) | Wheat (mg/mL) | Glucose |
|---|---|---|---|---|
| A | 0 | 2.5 | 7.5 | 60 |
| | 21 | 2.5 | 7.5 | 60 |
| | 30 | 2.5 | 7.5 | 60 |
| B | 0 | 5 | 5 | 60 |
| | 21 | 5 | 5 | 60 |
| | 48 | 5 | 5 | 60 |
| C | 0 | 10 | | 60 |
| | 21 | 10 | | 60 |
| | 30 | 10 | | 60 |
| | 48 | 10 | | 60 |
| Model system | Incubation time (min) | soy protein (mg/g model system) | wheat protein (mg/g model system) | |
| D | 0 | 5.5 | 49.5 | |
| | 10 | 5.5 | 49.5 | |
| | 15 | 5.5 | 49.5 | |
| E | 0 | 11 | 44 | |
| | 10 | 11 | 44 | |
| | 15 | 11 | 44 | |

VFDGELQEGR, from glycinin and NPFLFGSNR, from β conglycinin were detected in all the samples.

It is especially important to develop a method for the detection of cooked soy, as soy cannot be eaten uncooked due to the presence of trypsin and chymotrypsin inhibitors (Brandon and Friedman, 2002), although a contamination with raw soy may occur. It has previously been shown that food processing can reduce the antigenicity of soy proteins (Careri *et al.*, 2008). The antigenicity of soy proteins was significantly decreased by protein glycation (Houston *et al.*, 2010). Indeed, Babiker *et al*. showed that the allergenicity of P34, the immunodominant soy allergen was removed by the formation of a soy protein-galactomannan conjugate (Pedersen *et al.*, 2008).

### 2.4.6 Development of a mass-spectrometric method for quantification of soybean allergens

#### Comparison of uncooked and baked cookies spiked with increasing amounts of soy protein extract before baking

NPFLFGSNR, from β conglycinin could not be detected in cookies spiked at 50 ppm, after baking (20 minutes at 180 °C), and so this peptide was discontinued for the rest of the experiments. It is likely that it is modified or degraded, at least to some extent. It has previously been reported that glycinin is the more thermostable protein of the two, and so peptides from this protein are more likely to be detected after food processing (Mills *et al.*, 2003).

A calibration curve was made by the analysis of home-made cookies spiked at 0, 50 and 500, 2500 ppm soy protein extract before baking, as shown in Figure 41 (showing the first transition). The proportions of glycinin in the soy protein extract are constant, although the exact amount of glycinin spiked is unknown. If, for example, there is 30 % glycinin in soy, then the actual number of ppm of this allergen spiked would be 0, 15, 150 and 750 ppm. Baked and uncooked cookies made with four different amounts of spiked soybean protein extract were made.

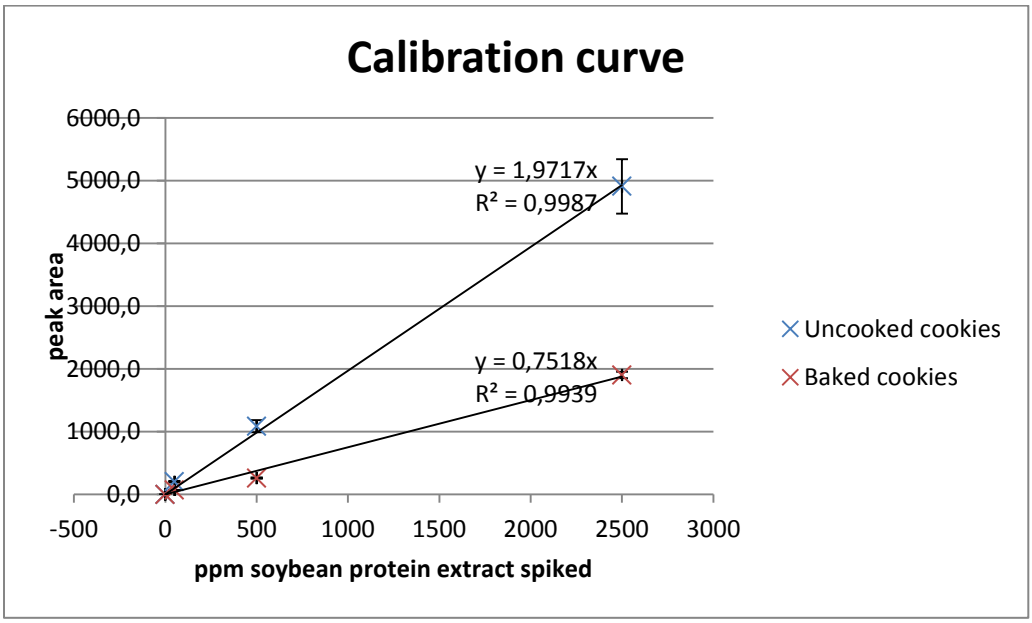


Figure 41 Calibration curves showing peak area as a function of ppm soybean protein extract spiked for uncooked or baked cookies

The peak intensities are similar for each of the different dough subsamples. Some variability in the samples may be observed because it is difficult to evenly distribute the soy throughout the dough. They are all samples from one portion of dough. It is likely that on a larger industrial scale this is not the case. The correct ion ratios were

obtained for the cookies with the matrix effect (between 1.41 and 2.11) with respect to the standard given in Table XXVII. For the uncooked cookies, the ion ratios were correct, and notably within the permitted 20 % error allowed by the European Commission decision 2002/657/EC. However, for the baked cookies, the LOD (limit of detection) was reached, as one of the samples, spiked with 50 ppm soybean protein extract did not have the correct ion ratio. For this matrix, the LOD is therefore attained. Therefore there must be some interference from the matrix at this concentration of matrix and soy. For the uncooked cookies, the ion ratios were correct for all the different concentrations tested. It is likely that reactions and degradation take place with the soy during baking. In uncooked soy, there are trypsin inhibitors, which may hinder the digestion step in the sample preparation. However, these may be inactivated due to a loss of native structure of the inhibitors, lost during the reduction and alkylation steps of the sample preparation.

Table XXVII Peak areas for cream chocolate dessert and biscuits

| Transitions | Soy cream dessert | Industrially made biscuit | Standard |
|---|---|---|---|
| 575.31>903.42 | 81285.33 ± 10509.83 | 564.67 ± 20.31 | 103683.67 ± 21575.80 |
| CV (%) | 12.93 | 3.60 | 1.98 |
| RT (min) | 4.67 ± 0.04 | 4.70 ± 0.03 | 4.68 ± 0.09 |
| S/N | 29516.18 ±4749.21 | 250.20 ±36.69 | 15289.08 ±838.43 |
| 575.31>788.39 | 46992.00 ± 6426.70 | 295.00 ± 7.00 | 58870.00 ± 11348.37 |
| CV (%) | 13.68 | 2.37 | 1.98 |
| RT | 4.67 ± 0.04 | 4.70 ± 0.04 | 4.68 ± 0.09 |
| S/N | 15485.19 ±2030.81 | 138.49 ±12.33 | 11495.18.397 ±2069.97 |
| Ion ratio | 1.73 ± 0.02 | 1.91± 0.08 | 1.76 ±0.03 |

The detection limits for different allergens in various food products need to be as low as possible (Poms *et al.*, 2005; Taylor *et al.*, 2004). It has been reported that only 41 to 200 mg of soy protein is necessary to provoke an allergic response in sensitive people (Ballmer-Weber *et al.*, 2007; Bindslev-Jensen *et al.*, 2002; Sicherer *et al.*, 2000; Wilson *et al.*, 2005).

Figure 41 shows the peak areas for the uncooked and baked cookies as a function of soy spiked. The method is sensitive, as shown by the detection of the peptide VFDGELQEGR in the home-made cookies spiked with 50 ppm of soybean protein extract. The final amount of soybean protein extract spiked must increase slightly after baking due to evaporation of water. The LOD has not been reached and remains to be determined for the uncooked cookies.

### Analysis of other real samples

Two industrially made products were analyzed: another type of biscuit and a soy cream dessert. The method works for both, as the ion ratios are within the 20% limit for the ion ratio, as described above. Table XXVII shows the peak areas for the two types of sample. The peak areas for the soy cream desserts are high, which is to be expected, due to the high concentration of soybean protein present. The method was also shown to be applicable for real samples as the ion ratios and retention times are correct. The industrial biscuit contains much less soy. From the peak areas for the triplicate of samples taken from the biscuits with respect to the peak areas in the calibration curve, the amount of soy protein extract spiked has been determined: equivalent of 732 ppm soy protein extract spiked approximately. The calibration curve for the home-made cookies can be used as an approximation of the amount of soy protein present. It must be taken into account when interpreting the data that different baking temperatures, times, and ingredients were used. By extrapolation of the calibration curve, the equivalent of approximately 11 % soy protein extract is present.

If the peptide is detected, then the presence of the allergen is confirmed, however this mass spectrometric method does not provide any information on the antigenicity of the soy proteins. These are preliminary results, that require a validation

## 2.5 Detection of hazelnut allergens in a standardized food matrix: lessons from the basophil activation test.

This study aims to confirm our observations with peanut in a hazelnut model (Sabato *et al.*, 2010a; Sabato *et al.*, 2010b).

For confirmation of our data obtained with peanut hazelnut was chosen, as there is an increasing prevalence of hazelnut allergy (EuroPrevall: http://www.europrevall.org/) and because we had the opportunity to validate a novel diagnostic tests that enables to establish a risk profile in hazelnut allergic (De Knop et al., Ped All Immunol 2010 in press). As it appeared that our patients with severe hazelnut allergy frequently demonstrate a concomitant allergy for egg's white and cow's milk, we could not rely upon traditional food to continue our analysis. Therefore, to circumvent these confounders, we applied different standardized matrices kindly provided by the Department of Food Safety and Food Quality, Research Group Food Chemistry and Human Nutrition & Department of Biochemistry and Microbiology, Laboratory for Protein Biochemistry and Biomolecular Engineering, Ghent University.

Basophils activation was performed as published before (Ebo et al., 2008; Ebo et al., 2007; Platteau et al., 2010)

The results of our experiments are summarized in the figures below. Figure 42 shows that basophils of patients with systemic reactions to hazelnut (n=6) can be clearly stimulated with hazelnut extracts (HZN UA and HA1). Pre-warming at 70°C during 24 h (HA2) and 48 h (HA3) has no influence on the activating capacity of hazelnut extract. This could be explained by the fact that 11S legumins (Cor a 9) are very heat stable. Even the addition of standardized amounts of wheat (HB1-3 and HC1-3) has little to no effect on the stimulatory effect of basophils of systemic hazelnut allergic patients. On the contrary, addition of glucose without or with heating at 70°C during 24-48 h (HE1-3, HD1-3 and HF1-3) seems to have some effect on these basophils.

Out of the results of this proof-of-concept can be concluded that a Mailliard reaction can have an influence on basophils of hazelnut allergic patients as compared with raw extracts. Results show that in 3 of the 5 patients still a basophil activation remains positive.

Figure 43 shows the results with same conditions on basophils of hazelnut allergic patients but with only a mild oral allergy syndrome (OAS: n=4). Basophils of this type of patients react on raw hazelnut extract (HZN UA and HA1). Even pre-warming at 70°C during 24 h and 48 h seems to have almost no influence on basophils of those patients. This finding is unusual because the major allergen of OAS (Cor a 1) is a thermo-labile antigen. A possible explanation could be that temperatures higher than 140°C are needed to denature hazelnut antigens. The addition of standardized amounts of wheat (HB1 and HC1), glucose (HD1) or both (HE1 and HF1) without pre-warming seems to have no effect. Nevertheless, with the same amounts of wheat and glucose added (HB to HF1-3) and pre-warming at 70°C cells could not be stimulated.

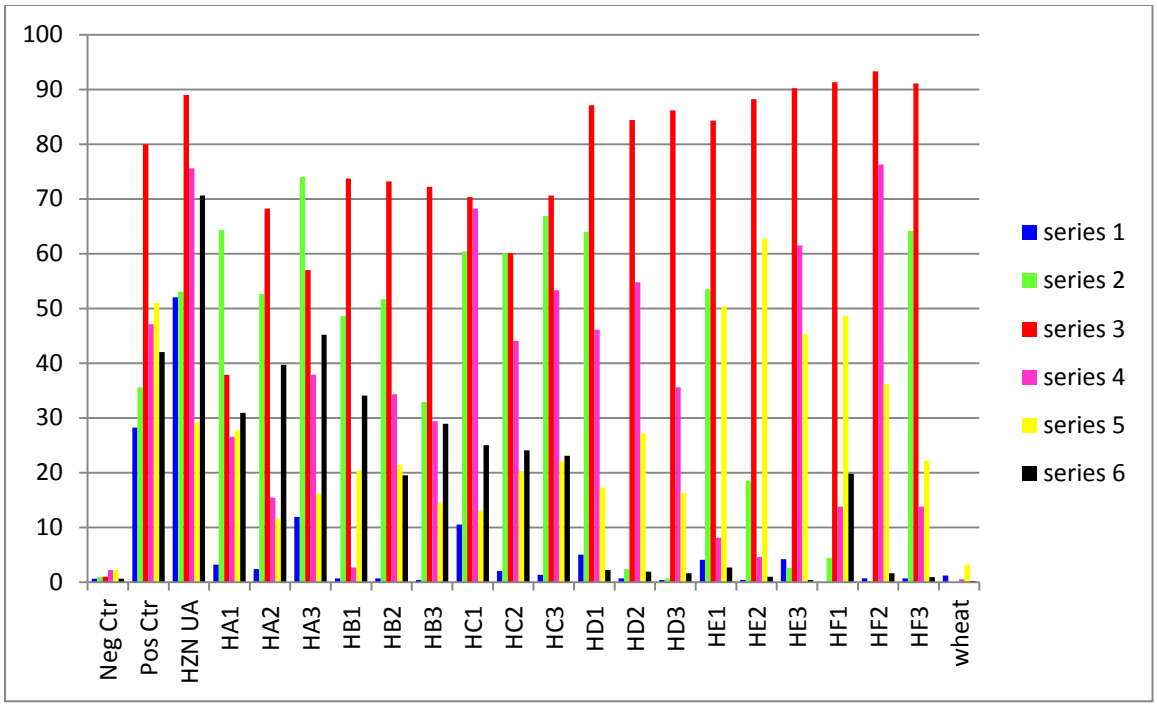


Figure 42 Basophil activation (expressed as percentage CD63 positive cells) in 6 patients with severe hazelnut allergy (series 1-6). Stimulation of cells with stimulation buffer (neg ctrl), anti-IgE as a positive control (pos ctrl) and 2 pure hazelnut extracts (HZN UA en HA1). Cells were also stimulated with hazelnut extract to which 2 different concentrations of wheat and/or glucose were added. HB and HC = addition of wheat, HD = addition of glucose, HE and HF = addition of wheat and glucose and finally wheat (as control). Experiments were performed without heating of extract (1) and after heating of extract (2 = 24h 70°C, 3 = 48h 70°C).

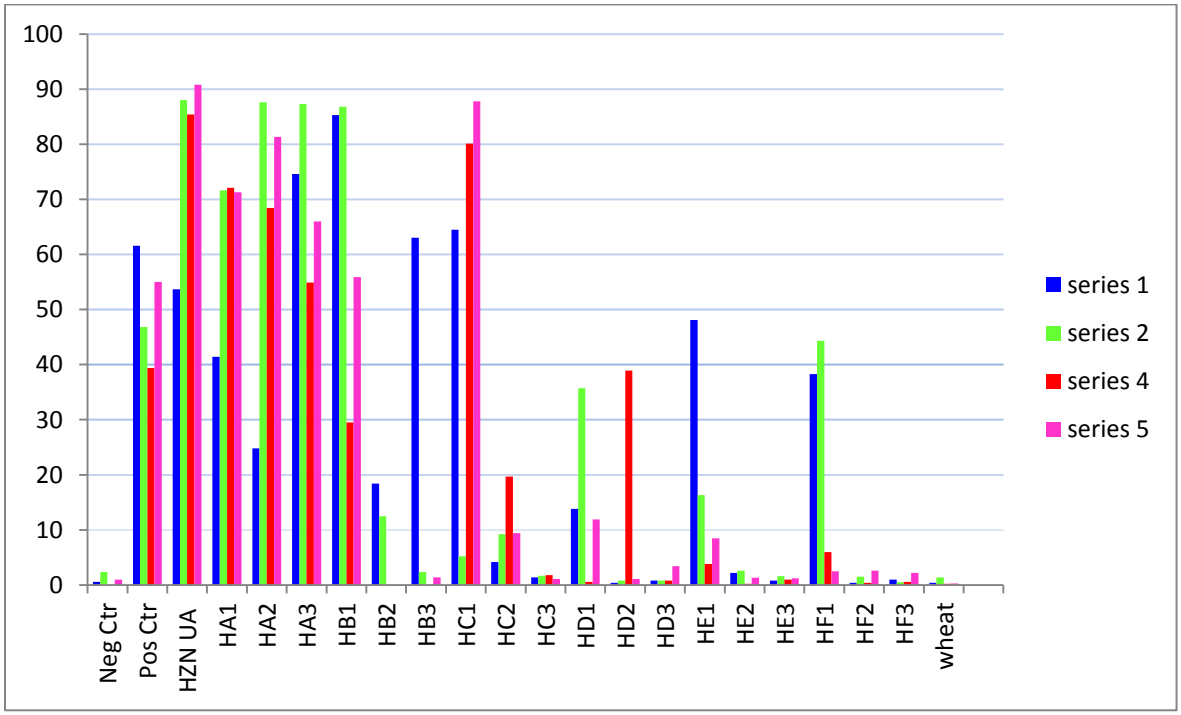


Figure 43 Basophil activation (expressed as percentage CD63 positive cells) in 4 patients with severe hazelnut allergy (series 1-4). Stimulation of cells with stimulation buffer (neg ctrl), anti-IgE as a positive control (pos ctrl) and 2 pure hazelnut extracts (HZN UA en HA1). Cells were also stimulated with hazelnut extract to which 2 different concentrations of wheat and/or glucose were added. HB and HC = addition of wheat, HD = addition of glucose, HE and HF = addition of wheat and glucose and finally wheat (as control). Experiments were performed without heating of extract (1) and after heating of extract (2 = 24h 70°C, 3 = 48h 70°C).

# 3 POLICY SUPPORT

Since Directive 2003/89/EC a EU legislation is in place on the labelling of food and food ingredients that contains allergenic compounds. This legislation describes a general framework which includes also a list of products which need to be labelled. This legislation has the goal to inform and protect allergic patients. But it also aims to set a legal framework which allows the companies to carry out their economic activities in a correct way. In order to support the implementation of this legislation a control plan consisting of a sampling plan and detection methods need to be in place.

During this project the performance of different detection methods has been evaluated. It is clear that different analytical approaches are currently available, going from DNA based methods, protein based methods making use of ELISA or MS/MS detection and biological testing systems, such as basophil activation tests. All have their pros and contras. An allergic reaction in a patient is induced by particular epitopes of for example a protein. It is however demonstrated that different allergic patients might react differently to a particular epitope. In other words, the reason why patients allergic for hazelnut show an allergic response might be caused by different epitopes. This fact and in addition the fact that the list in the legislation is ingredient-based has consequences for the detection methods to be used. In the ideal situation from the patients point of view he would be best served with a method which detects the allergy-causing agent (i.e. the epitopes). The consequence would be that the information should be personalised in function of the patient, which is in practise not feasible. Therefore, at the moment the method should be aiming to detect the ingredients used to produce the food. This is the case for both the PCR based methods, which detect specific elements of the genome of the products on the list and the ELISA based methods for which polyclonal antibodies are used that are raised against the total proteins extracted from a particular ingredient. In case of a positive result it should be interpreted that a particular ingredient is used that is on the list but it is not necessarily a prove that the epitope responsible for the allergic response is present in the food. In contrast, in case of a negative result it cannot be excluded that the target of detection is absent (e.g. the DNA fragment to be detected or the epitopes detected by the polyclonal antibodies used in the ELISA) while the epitope responsible for the allergic response is still present. However, the chance that this will happen is in our opinion low. In conclusion, both protein or DNA based methods are in line with the legislation aiming to label products based on the basis of ingredient used.

Another output of the project is that food processing will have an impact on extractability and/or detectability of the target of detection but also on the allergy-inducing capacity of an ingredient. During food processing some allergeny-inducing

epitopes might be lost, but it cannot be excluded that new allergy-inducing epitopes will be created.

Finally, from the producers point of view and the analytical point of view there is a strong need for thresholds for labelling. Those should be set such that they are relevant for the allergenic patients. More knowledge is needed to determine the correct thresholds. Linked to this there is also a strong need for harmonisation of the detection methods, correct interpretation of results obtained with methods detecting different targets, reference materials and monitoring planes. In order to organise this in a coordinated way at the European level we think that a coordinating network such as the European network for GMO Laboratories, which is running in the domain of GMO detection, would be very helpful.

# 4 DISSEMINATION AND VALORISATION

## 4.1 Poster presentations

Platteau, C., Bridts, C., Reybroeck, W., De Loose, M., Devreese, B., Daeseleire, E. Ebo, D. (2008) Comparison of three methods for the isolation of hazelnut proteins. 10th International Symposium on Immunological, Chemical and Clinical Problems of Food Allergy, Parma, Italy.

Fourdilis S., Bourgeon C., Kirsch S., Widart J., Maghuin-Rogister G., Scippo M.L. , De Pauw E. (2008) A new method for absolute quantification of allergens in food: the "Heavy Peptide" method. 10th International Symposium on Immunological, Chemical and Clinical Problems of Food Allergy, Parma (Italy)

Platteau, C., Taverniers, I., Daeseleire, E. and De Loose, M. (2009) Different methods to detect allergens in food. KVCV: Trends in Food Analysis VI, Ghent, Belgium.

Dobson R., Fourdilis S., Kirsch S., Baiwir D., Maghuin-Rogister G., Scippo M.L., De Pauw E.(2009) "Development of a quantitative method to detect trace amounts of hazelnut and soy allergens in food", NutrEvent

Platteau, C., Cucu, T., Daeseleire, E., De Meulenaer, B., De Loose, M., Taverniers, I. (2010) Influence of food processing on the detection of soy allergens by ELISA. Knowledge for Growth 6th edition, Ghent, Belgium

Platteau, C., Cucu, T., De Loose, M., De Meulenaer, B., Taverniers, I. (2010) ELISA detection of hazelnut allergens: influence of food processing. 16th PhD Symposium, Ghent, Belgium.

Cucu, T., De Meulenaer, B., Kerkaert, B., Vandenberghe, I. and Devreese, B. (2010) Stability of whey protein derived peptides upon severe protein glycation. *Open Innovation in Feed, Food and Health. Where Industry and academia meets!*, Ghent, Belgium

Cucu, T., Devreese, B., Mestdagh, F., Kerkaert, B., and De Meulenaer, B. (2010) Protein-lipid interactions during the incubation of whey proteins with autoxidizing lipids. *Open Innovation in Feed, Food and Health. Where Industry and academia meets!*, Ghent, Belgium

Dobson R., Fourdrilis S., Kirsch S., Baiwir D., Cucu T., De Meulenaer B., Maghuin-Rogister G., Scippo M.L., De Pauw E. (2010) Development of quantitative methods to detect trace amounts of hazelnut and soy allergens in food, 58th ASMS Conference

## 4.2 Oral presentations

Platteau, C., Cucu, T., De Loose, M., De Meulenaer, B. and Taverniers, I. (2010) The influence of food processing on the detection of hazelnut proteins. 6th European IAFP symposium, Dublin, Ireland.

Platteau, C., De Loose, M., De Meulenaer, B., Taverniers, I. (2010) Detection of hazelnut in food by PCR. 16th PhD Symposium, Ghent, Belgium.

Cucu, T.; De Meulenaer, B.; Kerkaert, B.; Vandenberghe, I.; Devreese, B. (2010). Effect of glycation in the presence or absence of soluble wheat proteins on major whey allergens. *Abstract of Scientific Papers of the 239th ACS National Meeting*, San Francisco, CA, USA.

Mestdagh, F.; Kerkaert, B.; Cucu, T.; De Meulenaer, B. (2010). Combined effect of light and omega-3 polyunsaturated fatty acid fortification on the oxidative stability of milk proteins. *Abstract of Scientific Papers of the 239th ACS National Meeting, San Francisco*, CA, USA.

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