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# Sensitive time-resolved fluoroimmunoassay for the detection of hazelnut (*Corylus avellana*) protein traces in food matrices

C.K. Fæste\*, L. Holden, C. Plassen, B. Almli

National Veterinary Institute, PO BOX 8156, dep., N-0033 Oslo, Norway

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## Abstract

Allergy to hazelnut is one of the most prevalent causes of severe food-allergic reactions in Norway, as recorded by The Norwegian National Reporting System and Register of Severe Allergic Reactions to Food. In the majority of the reported cases, there is “hidden”, unlabelled hazelnut protein in processed foods like chocolate, cookies and cereal mixtures the eliciting agent. For a food survey study performed to evaluate the labelling practices with regards to hazelnut on behalf of the Norwegian Food Safety Authority, a new sensitive time-resolved fluoroimmunoassay (TR-FIA) for the detection of hazelnut protein traces in food matrices was developed and validated. The unique fluorometric properties of the europium-chelates used improved the signal-to-noise ratio because of low matrix interference and led to an enhanced sensitivity. The limit of detection was 0.1 mg/kg and the limit of quantitation was 0.33 mg/kg hazelnut protein. The recovery ranged from 73% to 123% in cookies and cereals, and from 50 to 77% in chocolate. The intra-assay precision was 7% and the inter-assay precision was 19%. Of 100 randomly chosen retail food products in Norway labelled “may contain hazelnut”, 36 contained <0.2 mg/kg and seven >10 mg/kg hazelnut protein, demonstrating how differently this precautionary label is used.

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## 1. Introduction

Food allergy to hazelnut (*Corylus avellana*) has caused several incidents of severe allergic reactions (Wensing et al., 2002). In Europe, hazelnut has been included into a list of 11 major food allergens and products thereof which have to be mandatorily declared on food products, independently from other labelling policies (European Commission, 2002).

The allergenic proteins identified in hazelnut so far have been found to be thermolabile (Cor a 1, Cor a 2)

(Hansen et al., 2003) or thermostabile (Cor a 8, Cor a 9, Cor a 10, Cor a 11) (Pastorello et al., 2002) and seem to differ in their immunogenicity. However, analytical methods for the detection of hazelnut in foods should be able to identify hazelnut proteins in all modifications caused by processing.

Hazelnuts are used in a large variety of confectionery foods like chocolates, nougat, cookies, cereal mixtures, muesli bars and chocolate spreads. Due to manufacturing practices, e.g., shared equipment, or by accidental introduction under the preparation of a meal, cross-contamination of originally hazelnut-free food may occur. Undeclared hazelnut traces represent an acute danger to sensitised persons (Vieths, 2003). In some cases, even

\* Corresponding author. Tel.: +47 23216232; fax: +47 23216201.  
E-mail address: [christiane.faste@vetinst.no](mailto:christiane.faste@vetinst.no) (C.K. Fæste).

minute amounts of hazelnut protein are enough to trigger severe allergic reactions. As defined by double-blind placebo-controlled food studies (DBPCFC), the minimum provoking doses in hazelnut-allergic patients range from 1 to 100 mg hazelnut protein (Wensing et al., 2002).

Analytical methods for the detection of hazelnut in foods are requested by manufacturers, authorities and consumer organisations. The first immunochemical assays developed were used to determine the hazelnut content in hazelnut-containing products (Mohr et al., 1983; Klein et al., 1985) and had detection limits > 100 mg hazelnut protein/kg food. In contrary, recent methods are designed for the detection of trace amounts and show considerably improved sensitivities. The LODs for the respective methods, all but the last one reported in amount hazelnut protein/kg food, are 20 mg/kg by rocket immunoelectrophoresis analysis (Malmheden Yman et al., 1994), 5 mg/kg by SDS-electrophoresis/immunoblot with chemoluminescence detection (Scheibe et al., 2001), 10 mg/kg by a Biosensor-based test (Jonsson, 2002), and 1 mg/kg (Koppelman et al., 1999), 0.6 mg/kg (Holzhauser and Vieths, 1999), 1 mg/kg (Blais and Phillippe, 2001), 0.3 mg/kg (Ben Rejeb et al., 2003) and 0.2–1.2 mg whole hazelnut/kg (Kiening et al., 2005) by enzyme-linked immunosorbent assay. With DNA-based polymerase chain reaction (PCR), the LOD was 10 mg hazelnut/kg (Holzhauser et al., 2000) and 4 mg hazelnut/kg with a combined PCR-ELISA (Holzhauser et al., 2002) which is commercially distributed. Currently, four commercial ELISA-kits with nominal LODs of 1.5 or 2.5 mg hazelnut/kg, and 0.5 or 0.8 mg/kg hazelnut protein in food are available.

Time-resolved fluorometry and lanthanide labels have been utilised for immunoassays for some time (Soini and Lovgren, 1987). Several dissociation enhanced lanthanide fluoroimmunoassays (DELFI) measuring cytokines and plasma proteins for diagnostic reasons have been described (Hemmila, 1988). Generally, the lanthanide ion-chelate which is covalently bound to an antibody is dissociated by pH-shift into a new highly fluorescent chelate after binding to the target protein. Most frequently used is the europium ion ( $\text{Eu}^{3+}$ ) in connection with several different complex chelators. The special fluorescence properties of the lanthanide chelate labels, such as long decay times, large Stokes' shifts between excitation and emission wavelengths, sharp emission peaks and high fluorescence intensities, entail low matrix interferences and allow the development of very sensitive detection methods.

In Norway, hazelnut allergy is responsible for a considerable number of anaphylactic incidents (Løvik et al., 2004). The objectives of this study were to develop and

validate a sensitive and specific assay for the detection of hazelnut traces in foods and to survey commercial food products labelled "may contain hazelnut". To reach this aim, we chose lanthanide immunofluorometry as means of detection.

## 2. Materials and methods

### 2.1. Hazelnut protein standard and sample preparation

For the preparation of a hazelnut protein standard, raw Mina hazelnuts (Iran) were homogenised with a blade homogenisator (Grindomic GM200, Retsch GmbH, Haan, Germany), suspended in extraction buffer (0.1 M Tris/0.5 M glycine buffer pH 8.7) at a ratio of 1:100, vortexed rigorously for 30 s, extracted at room temperature (RT) for 30 min, centrifuged at 15,000×g, 4 °C for 25 min and filtered through glass wool. After determination of the total protein content with the Lowry method (DC protein assay, Bio-Rad, Hercules, CA), the protein standard solution was diluted with phosphate-buffered saline (PBS pH 7.4, Oxoid, Basingstoke, UK) to 2 mg/ml and stored at –20 °C in aliquots.

All food samples were purchased from local stores. They were homogenised and extracted in the extraction buffer at a ratio of 1:5 overnight in a shaking water bath (45 °C) and centrifuged as described above. Extracts not totally cleared by centrifugation were additionally filtered through glass wool. Several hazelnut-free products were used as negative controls and for standard addition in recovery experiments. For the survey study, samples from two different lots of various cereal mixtures (34), cookies (32) and chocolates (34) were analysed in triplicates.

### 2.2. Antibody purification and europium-chelate labelling

A polyclonal rabbit antiserum, raised against the corylin fraction from native hazelnut protein and immunoabsorbed against a variety of different nut proteins by the manufacturer (no. 45261, Riedel de-Haen, Seelze, Germany), was used in the TR-FIA. The IgG-fraction was isolated by using a HiTrap rProtein A affinity column (no. 17-5070-01, Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions, and 1 mg of the purified antibody was sent to the Wallac Labelling Service (PerkinElmer Life Science, Turku, Finland) to be labelled with the  $\text{Eu}^{3+}$ -chelate of  $N^1$ -(4-isothiocyanatobenzyl)-diethylenetriamine- $N^2, N^3, N^4$ -tetrakis(acetic acid) [Eu(III)-N1 ITC]. The labelling reaction was performed at pH 9, 4 °C overnight in a

100 mM Na<sub>2</sub>CO<sub>3</sub> buffer, and the Eu<sup>3+</sup>-labelled antibody was purified by gel filtration on a Sephadex G50-column and eluted with a 50 mM Tris–HCl buffer, containing 0.9% NaCl and 0.05% NaN<sub>3</sub>. A total amount of 0.93 mg Eu<sup>3+</sup>-labelled anti-corylin (6 Eu<sup>3+</sup>/protein molecule) was obtained. The purified unlabelled (5 mg/ml) and the Eu<sup>3+</sup>-labelled (117 µg/ml, 0.73 µmol/l) anti-corylin antibodies were both stored in aliquots at –20 °C until use.

### 2.3. Western blot analysis

The NuPAGE Gel System (Invitrogen, Carlsbad, CA) was used for electrophoretic separation of protein samples by SDS-PAGE, in accordance to the manufacturer's instructions. Separation was performed under reducing conditions for 40 min at 200 V in 2-(*N*-morpholino)ethane sulfonic acid (MES) SDS running buffer, using 4–12% Bis–Tris gels and SeeBluePlus2 pre-stained reference standard. Samples were prepared with lithium dodecyl sulphate (LDS) sample buffer and dithiothreitol (DDT) reducing agent (all from Invitrogen). The proteins were electrophoretically transferred from the gel onto a nitrocellulose membrane (Bio-Rad, Hercules, CA) for 60 min at 30 V with transfer buffer in an XCell II Blot Module (Invitrogen). Tris-buffered saline (TBS) containing 0.1% Tween 20 pH 7.6 was used as washing buffer, and after addition of 1% BSA as blocking and assay buffer for the Western blots. After blocking for 30–60 min, the blot was incubated overnight at 4 °C with the unlabelled anti-corylin antibody (5 mg/ml) diluted 1:500,000 in assay buffer. The blot was washed (3 × 15 min) and incubated for 1 h with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (Zymed, San Francisco, CA) diluted 1:5000 in assay buffer. After washing (3 × 10 min), the membrane was developed with 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution (Zymed) until bands of satisfactory intensity appeared (2–10 min). All washing and incubation steps were performed at RT under gentle shaking.

### 2.4. Procedure of the hazelnut TR-FIA

Flat-bottom DELFIA®-yellow 96-well plates (no. AAAND-0001, DELFIA®-yellow plate, PerkinElmer, Turku, Finland) with high protein binding capacity and low fluorescence background were coated (100 µl/well) with 5 µg/ml of the unlabelled polyclonal anti-corylin antibody at 4 °C overnight, after diluting the antibody stock solution in coating buffer (0.05 M carbonate–bicarbonate buffer pH 9.8, Sigma-Aldrich, Oslo, Norway). After washing (Skan Washer 400, Skatron Instruments, Lier, Norway) with phosphate-buffered

saline (PBS) containing 0.05% Tween 20 as washing buffer, the plates were blocked by incubation with 250 µl/well of blocking/assay buffer (1% BSA in PBS) for 1 h at RT. After further washing, the plates were ready-to-use and could be stored at 4 °C for several weeks.

For the determination of hazelnut in foods, a coated plate was room-tempered and hazelnut protein standard, buffer blanks, matrix blanks, control samples and food samples were added (100 µl/well) in triplicates. The hazelnut protein standard was added in a concentration of 5 µg/ml and applied in twofold serial dilutions in blocking/assay buffer, resulting in a concentration range from 5 to 0.005 µg/ml. The control and food samples were diluted 1:4 in blocking/assay buffer before application, according to dilution tests performed by adding standard protein to blank matrices. Samples containing high hazelnut protein contents, as in the recovery study, were diluted 1:40. The plate was incubated for 1 h at 37 °C in an incubator (Series 4000, Termaks AS, Bergen, Norway). After washing, 100 µl of the Eu<sup>3+</sup>-labelled polyclonal anti-corylin antibody (diluted with blocking/assay buffer to 300 ng/ml) were added. The plate was incubated for 1 h at RT under gentle shaking (Shuttler MTS 4I, IKA-Werke GmbH, Staufen, Germany), followed by a final washing step. For the development of the fluorescence signal, 200 µl of DELFIA®-Enhancement solution (B118-100, PerkinElmer, Turku, Finland) were added to each well. The plate was incubated for 10 min at RT under gentle shaking to develop maximal signal intensity, and the Eu<sup>3+</sup>-fluorescence emission was measured at 615 nm with a 1420 Victor<sup>2</sup> multilabel counter (EG&G Wallac, Oslo, Norway). As Eu<sup>3+</sup>-fluorescence control, a 1 nmol/l europium standard solution (no. B119-100, PerkinElmer, Turku, Finland) with a reference intensity of 1,000,000 counts/nmol was used.

Under the development of the method, two other coating buffers were evaluated, a buffer recommended in the DELFIA-manual (50 mM Tris–HCl pH 7.8 (9 g/l NaCl, 0.5 g/l NaN<sub>3</sub>, 5 g/l BSA, 0.5 g/l bovine g-globulin, 0.1 g/l Tween 40, 20 µM diethylenetriamine pentaacetic acid, 20 mg/l cherry red) and PBS. They both gave inferior results compared to the TR-FIA procedure described above which was used in all the following experiments.

### 2.5. Assay validation: specificity, precision, accuracy, limit of detection and limit of quantitation

The in-house validation of the TR-FIA for the detection of hazelnut protein traces in food matrices followed generally the recommendations in the “Harmonized

guidelines for single-laboratory validation of methods of analysis” (Thompson et al., 2002).

The specificity of the method was tested in cross-reactivity studies. Extracts from various nuts and stonefruits (almond (*Prunus dulcis*), walnut (*Juglans regia*), Brazil nut (*Bertholletia excelsa*), pecan nut (*Carya illinoensis*), cashew nut (*Anacardium occidentale*), macadamia nut (*Macadamia integrifolia*), pistachio (*Pistacia vera*), coconut (*Cocos nucifera*), edible chestnut (*Castanea sativa*)) and legumes (soybean (*Glycine max*), lupine (*Lupinus albus*), peanut (*Arachis hypogaea*), chickpea (*Cicer arietinum*), common pea (*Pisum sativum*), white bean (*Phaseolus vulgaris*), lentil (*Lens culinaris*)) and seeds (pine seed (*Pinus pinea*), sesame seed (*Sesamum indicum*), pumpkin seed (*Curcubita pepo*)) were prepared as described before from food bought at local stores. Other main food allergens (ovalbumin (*Gallus domesticus*), ovomucoid (*Gallus domesticus*), bovine casein (*Bos domesticus*) (all from SigmaAldrich, NY, USA), red shrimp tropomyosin (*Pandalus borealis*), Atlantic cod parvalbumin (*Gadus morhua*), celery (*Apium graveolens*), mustard (*Sinapis alba*) (all purified according Fæste et al., 2003) were also included into the test. All extracts were tested in the hazelnut TR-FIA at a concentration of 100 µg/ml total protein, and potentially cross-reactive foods were further investigated with Western blot.

The precision of the method was determined by using a chocolate cookie and a milk chocolate containing less than 4 mg hazelnut protein/kg, which had been previously confirmed by semi-quantitative Western blot analysis. After extraction, the samples were stored in aliquots at –20 °C, and each analysis was performed with freshly thawed extract. The intra-assay precision was determined as the mean coefficient of variation (CV) of 20 replicates;

the inter-assay precision was calculated as the mean CV based on triplicate analyses on 15 different days.

The accuracy of the method was evaluated by performing recovery studies using four different blank matrices. In six experiments, hazelnut-free cereals, cookies, milk chocolate and dark chocolate were homogenised to fine powders and spiked with the hazelnut protein standard at levels of 0, 1, 25 and 150 mg/kg sample by adding 1 ml of suitably diluted standard solution to 2 g of the respective food. After equilibration for 15 min at RT, additional 9 ml extraction buffer were added, and the extraction was performed as described previously. The samples were analysed in triplicate, and the mean values for the recoveries and the standard errors of the means were calculated. The addition of 5% fish gelatin (Sigma-Aldrich, Oslo, Norway) or 1% dry milk powder to the extraction buffer for the preparation of chocolate-containing samples was evaluated.

The LOD of the hazelnut TR-FIA was calculated as the mean of the measured content of 23 buffer blank samples plus three times the standard deviation (S.D.) of the mean value. The limit of quantitation (LOQ) was determined as the mean of the measured content of 23 buffer blank samples plus 10 times the standard deviation (S.D.) of the mean value.

### 3. Results

#### 3.1. Detection of hazelnut protein in various foods

The protein-binding characteristics of the polyclonal anti-corylin antibody were determined on Western blot, using samples containing both raw and processed hazelnut protein (Fig. 1A). In the hazelnut protein standard

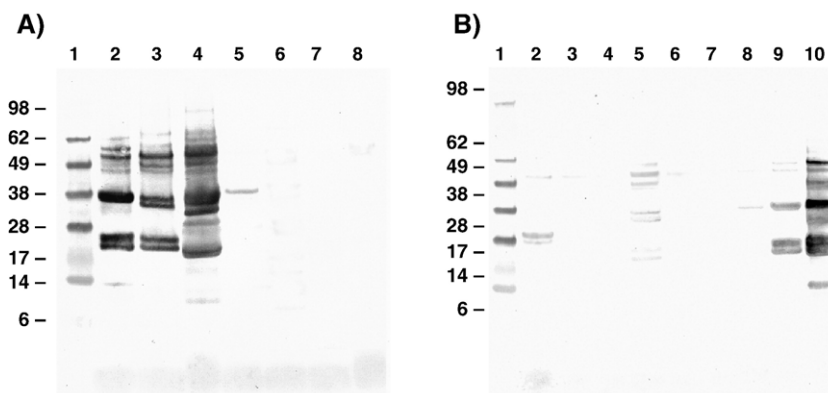


Fig. 1. Western blot analysis of various foods using purified polyclonal anti-hazelnut antibody. Lane 1 in panels A and B: molecular weight marker, protein sizes (kDa) are indicated on the left side of the gels. (A) Detection of corylin in foods typically containing hazelnut (H) and in matrix blanks. Lane 2: hazelnut protein standard; lane 3: cookie/H; lane 4: cereals/H; lane 5: dark chocolate/H; lane 6: cookie; lane 7: cereals; lane 8: dark chocolate. (B) Potentially cross-reactive foods. Lane 2: 10 µg coconut; lane 3: 1 µg coconut; lane 4: 0.1 µg coconut; lane 5: 10 µg walnut; lane 6: 1 µg walnut; lane 7: 0.1 µg walnut; lane 8: 0.1 µg hazelnut; lane 9: 1 µg hazelnut; lane 10: 10 µg hazelnut.

(lane 2), protein bands of major intensities were detected at 19, 23 kDa and 38 kDa and bands of minor intensities were detected at 14, 48, 53, 58 and 62 kDa. The protein patterns detected in the cookie (lane 3) and the cereals (lane 4) were very similar to the standard, though some extra bands were visible and the 14 kDa band was missing. Only one protein at 38 kDa was detected in the dark chocolate (lane 5). In the extracts of matching blank food matrices, no IgG-binding was observed (lanes 6–8).

### 3.2. Standard curve of the hazelnut TR-FIA

The hazelnut protein standard was used in a standard curve with concentrations from 0.005 to 5 µg/ml. The working range of the assay which was defined as the part of the curve which had a correlation coefficient of  $r^2 > 0.99$  when applying linear regression ranged from 0.005 µg/ml to 0.32 µg/ml (Fig. 2). For the evaluation of possible matrix effects, standard curves were prepared in different dilutions of blank cookie and chocolate matrix extracts. The results obtained were compared to those of the standard curve prepared in blocking/assay buffer. Whereas undiluted matrix extracts quenched the fluorescence signal with about 20–50% and caused increased background signals at lower concentrations, there was negligible interference at 1:4 dilution and none at 1:10 where a difference to the standard curve in buffer was not longer observable (data not shown). Therefore, all food extracts were pre-diluted 1:4 and, if necessary, additionally diluted twice twofold on the plate.

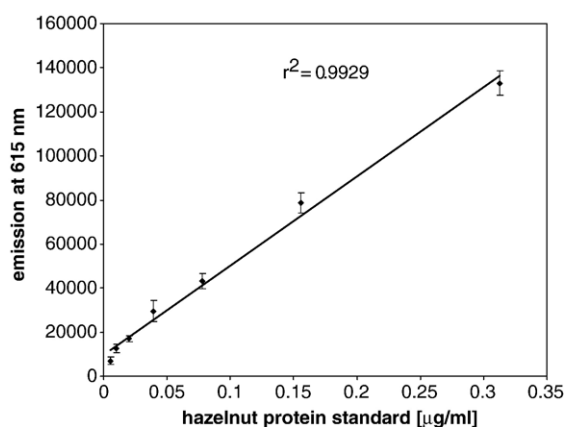


Fig. 2. Representative seven-point calibration curve, based on the standard curve obtained using the hazelnut protein standard in the hazelnut TR-FIA. The graph shows the average values of triplicate measurements, the standard deviations, and the linear regression curve and coefficient  $r^2$ .

Table 1

Cross-reactivity of nuts, legumes, seeds and other food ingredients in the hazelnut TR-FIA

| Food/ingredient | Recovery hazelnut protein (%) |
|-----------------|-------------------------------|
| Almond          | <0.00001                      |
| Brazil nut      | <0.00001                      |
| Cashew nut      | <0.00001                      |
| Chestnut        | <0.00001                      |
| Coconut         | 0.00004                       |
| Macadamia nut   | <0.00001                      |
| Pecan nut       | <0.00001                      |
| Pistachio       | <0.00001                      |
| Walnut          | 0.00011                       |
| Chickpea        | <0.00001                      |
| Common pea      | <0.00001                      |
| Lentil          | <0.00001                      |
| Lupine          | <0.00001                      |
| Peanut          | <0.00001                      |
| Soybean         | <0.00001                      |
| White bean      | <0.00001                      |
| Pine seed       | <0.00001                      |
| Pumpkin seed    | <0.00001                      |
| Sesame seed     | <0.00001                      |
| Casein          | <0.00001                      |
| Celery          | <0.00001                      |
| Mustard         | <0.00001                      |
| Ovalbumin       | <0.00001                      |
| Ovomucoid       | <0.00001                      |
| Parvalbumin     | <0.00001                      |
| Tropomyosin     | <0.00001                      |

### 3.3. Specificity

Extracts of various nuts, legumes, seeds and several ubiquitous food ingredients were tested for cross-reactions in the hazelnut TR-FIA (Table 1). Using 100 µg/ml of the different proteins, positive signals were only detected for walnut and coconut, resulting in 0.00011% (1.1 mg/kg) and 0.00004% (0.4 mg/kg) recovery of hazelnut protein, respectively. Further investigations with Western blot (Fig. 1B), applying equal protein amounts of walnut and coconut, showed that the protein bands in the walnut and coconut extracts which were detected by the polyclonal anti-corylin antibody differed in size and intensity from the band pattern in the hazelnut protein standard.

### 3.4. Precision

For the determination of the intra-assay and inter-assay precision of the hazelnut TR-FIA, two representative foods containing less than 4 mg/kg hazelnut protein, a chocolate cookie and a milk chocolate, were analysed. The intra-assay precision, as expressed by the coefficient of variation (% CV) was 8.4% for the chocolate cookie and 7.2% for the milk chocolate. The

Table 2

Intra- and inter-assay variances (% CV) determined for the hazelnut TR-FIA using hazelnut-containing foods

| Food           | Hazelnut protein (mean mg/kg) | Intra-assay variance (% CV) (n=20) | Inter-assay variance (% CV) (n=15) |
|----------------|-------------------------------|------------------------------------|------------------------------------|
|                |                               |                                    |                                    |
| Chocolate      | 1.55                          | 8.4                                | 20                                 |
| cookie         |                               |                                    |                                    |
| Milk chocolate | 3.29                          | 7.2                                | 19                                 |

The intra-assay variances were calculated from 20 replicates and the inter-assay variances were calculated from triplicate analysis on 15 different days.

inter-assay precision was determined as 20% and 19%, respectively (Table 2).

The DELFIA-assay buffer and the PBS buffer, which both were alternatively tested as potential coating buffers under method development, gave considerably poorer % CV data in the precision studies (data not shown).

### 3.5. Accuracy

Four blank samples from foods matching typically hazelnut-containing matrices, i.e., cereals, cookies, milk chocolate and dark chocolate, were spiked with low, intermediate and high concentrations of the hazelnut standard protein. After sample extraction, recovery rates were determined in the hazelnut TR-FIA (Table 3). In cereals and cookies, the recoveries ranged from 73% to 123%. In milk chocolate and dark chocolate, the recoveries ranged from 50% to 77%. No difference between low and high level hazelnut protein concentrations was observed. Fish gelatin in the extraction buffer did not increase the recovery rates of hazelnut protein from the chocolate samples. However, the addition of dry milk increased the recoveries from chocolate matrix to  $\geq 100\%$ .

Two additional assay performance studies were done. The samples from the food survey were re-determined

Table 3

Recovery (%) of hazelnut protein from various blank food samples spiked with 1, 25 or 150 mg/kg of hazelnut protein standard<sup>a</sup>

| Blank food                  | Recovery (%) at indicated amount of hazelnut protein added in mg/kg |             |             |
|-----------------------------|---------------------------------------------------------------------|-------------|-------------|
|                             | 1                                                                   | 25          | 150         |
| Cereals                     | 105 $\pm$ 16                                                        | 77 $\pm$ 8  | 123 $\pm$ 7 |
| Cookies                     | 97 $\pm$ 4                                                          | 73 $\pm$ 10 | 81 $\pm$ 8  |
| Milk chocolate <sup>b</sup> | 71 $\pm$ 4                                                          | 50 $\pm$ 11 | 63 $\pm$ 6  |
| Dark chocolate <sup>b</sup> | 77 $\pm$ 8                                                          | 54 $\pm$ 1  | 67 $\pm$ 7  |

<sup>a</sup> Values represent the average of six spiking experiments and are reported as mean $\pm$ standard error of the mean (S.E.M.).

<sup>b</sup> By addition of 1% dry milk to the extraction buffer, the recoveries increased to  $\geq 100\%$ .

Table 4

Survey study on foods labelled “may contain hazelnut” in Norway

| Food product type | Number of products analysed <sup>a</sup> | Hazelnut protein content (mg/kg) |       |      |     |
|-------------------|------------------------------------------|----------------------------------|-------|------|-----|
|                   |                                          | <0.2                             | 0.2–1 | 1–10 | >10 |
| Cereals           | 34                                       | 21                               | 10    | 3    | 0   |
| Cookies           | 32                                       | 24                               | 6     | 2    | 0   |
| Milk chocolate    | 26                                       | 8                                | 1     | 11   | 6   |
| Dark chocolate    | 8                                        | 4                                | 0     | 3    | 1   |

<sup>a</sup> All food products were purchased from local stores in 2004. Two different lot numbers of each product were analyzed as described in “Materials and Methods”.

by using rocket electrophoresis (LOD 20 mg/kg), and control samples from an inter-laboratory validation study of two commercial hazelnut ELISA-kits were successfully re-analysed with the new hazelnut TR-FIA (data not shown).

### 3.6. Limit of detection and limit of quantitation

The LOD of the hazelnut TR-FIA was 0.005  $\mu$ g/ml hazelnut standard protein in the assay, equivalent to 0.1 mg/kg hazelnut protein in food, considering the sample dilution in the assay. The LOQ was 0.016  $\mu$ g/ml hazelnut standard protein in the assay and 0.33 mg/kg hazelnut protein in food.

### 3.7. Survey study on foods labelled “may contain hazelnut” in Norway

In total, 100 representative food products, i.e., 50 branded articles with two lot numbers each, were purchased from local stores in 2004 (Table 4). The chocolate-containing products showed a considerable higher contamination with hazelnut than other kinds of food. 42% of the milk chocolates contained 1–10 mg/kg and 23% >10 mg/kg hazelnut protein. The highest concentration measured was 44 mg/kg. 38% of the dark chocolates contained 1–10 mg/kg and 12% >10 mg/kg hazelnut protein. The highest concentration measured was 17 mg/kg hazelnut protein. In contrary, only 9% and 6% of the cereals and cookies, respectively, contained 1–10 mg/kg hazelnut protein, none was >10 mg/kg. The highest concentrations measured were 3.4 mg/kg and 8.9 mg/kg, respectively.

## 4. Discussion

Contamination of food is probably the main reason why sensitised consumers ingest hidden allergens. Food manufacturing and hygiene practices vary, and contaminations may occur due to shared equipment or production lines, re-circulating of intermediate products,

inadequate cleaning routines or accidental introduction of hazelnut traces under the preparation of a meal (Fæste et al., 2003). Furthermore, raw materials used in the manufacturing process may already contain proteins with an allergenic potential. Hazelnut is a common ingredient in various foods and consequently a frequent hidden allergen, possibly dangerous for hazelnut-allergic persons. Anaphylactic reactions caused by hazelnut can already be elicited by low amounts of hazelnut protein (Wensing et al., 2002).

Hazelnut is one of the “big eight” major food allergens (Yunginger, 1990). Food authorities in different countries are therefore interested to monitor the frequency and severity of hazelnut allergy, to survey the distribution of hazelnut traces in foods, to follow the food labelling practices, and to support the development of analytical methods for the detection of hazelnut protein at low levels in food matrices. A number of hazelnut assays has already been published (Poms et al., 2004), however, by introducing new techniques or detection systems even higher sensitivities might be achieved.

The hazelnut TR-FIA presented here uses lanthanide fluorometry to achieve high sensitivity. By labelling the detection antibody with  $\text{Eu}^{3+}$ -chelates, the analysis can be performed time-resolved which gives the advantage of temporal and spectral resolution. The long decay time of the  $\text{Eu}^{3+}$ -fluorescence label exceeds 1,000,000 ns, compared to 10 ns of non-specific background fluorescence, considerably reducing the signal-to-noise ratio. In the method described here, the fluorescence is measured for 400  $\mu\text{s}$  after a delay time of 400  $\mu\text{s}$ . This is about 200,000 times longer than for any conventional, non-lanthanide fluorescent. Characteristic for  $\text{Eu}^{3+}$ -fluorescence is the big difference between excitation (340 nm) and emission (613 nm) wavelengths which surpasses these of other lanthanides like terbium (Tb), dysprosium (Dy) and samarium (Sm). A wide Stokes' shift like this leads to singularly high sensitivity of the fluorescence measurement. Additionally, the narrow emission peak contributes to increase the signal-to-noise ratio. In the DELFIA assay, the  $\text{Eu}^{3+}$ -chelate is dissociated into a new highly fluorescent chelate inside a protective micelle, triggered by a pH-shift that increases the fluorescence intensity furthermore. All effects combined result in a sensitive mode of detection, which can be utilised for immunochemical analysis. Measuring instruments like, e.g., the Victor<sup>2</sup> counter are able to determine 10 amol  $\text{Eu}^{3+}$ /well in an observation time of 2 min/96-well plate.

For the construction of the hazelnut TR-FIA, a commercially available polyclonal anti-corylin antibody was used for capture and an  $\text{Eu}^{3+}$ -chelate conjugate of the same

antibody was used for detection. Although monoclonal antibodies in general might give higher sensitivity towards their target proteins, a polyclonal antibody has the potential to recognise various epitopes on different proteins. This versatility was considered advantageous for the intended application of the TR-FIA and, in fact, the antibody chosen recognised characteristic corylin bands (Holzhauser and Vieths, 1999; Pastorello et al., 2002) on Western blot, both in raw and processed foods. A 38 kDa protein, possibly the seed storage protein Cor a 9 (Roux et al., 2003), was detected in the hazelnut protein standard and all hazelnut-containing samples. Even if processing like roasting may alter the protein composition in hazelnuts by denaturing specific proteins like Cor a 1 and Cor a 2 (Hansen et al., 2003), a polyclonal antibody raised against native corylin still detects enough epitopes to allow positive identification as also has previously been shown (Klein et al., 1985; Holzhauser and Vieths, 1999).

The hazelnut standard protein used in the hazelnut TR-FIA originated from raw Iranian Mina hazelnuts, containing an extractable protein amount of 19.9%. However, considering the varying protein content in the different cultivars (Tous et al., 1994), protein extractabilities which can differ by a factor of 2–3 depending, e.g., on the degree of processing (Kiening et al., 2005), and the lack of certified authentic reference material, the application of a correlation factor for the evaluation of the amount of whole hazelnuts in a food was thought to be unserviceable with respect to possible under- or overestimations.

The standard curve of the hazelnut TR-FIA is linear at hazelnut protein concentrations as low as 0.005  $\mu\text{g}/\text{ml}$  and is proportionally over three decades of concentration. The high sensitivity allows the determination of minute traces of hazelnut protein in foods, since the  $\text{Eu}^{3+}$ -fluorescence signal is only quenched by undiluted matrix extracts. When using a 1:4 or 1:10 dilution, this interference was negligible or completely removed, showing the advantages of the lanthanide-fluorometric detection method.

The specificity of the anti-corylin antibody was studied in the hazelnut TR-FIA by cross-reaction experiments with extracts from nuts, legumes, seeds and some typical food ingredients. Cross-reactivity was detected only for walnut and coconut with 0.00011% and 0.00004% recovery, respectively. Western blot analysis could be used for additional confirmation if hazelnut traces have to be detected in foods containing high amounts of walnut or coconut. A reduced selectivity of the anti-hazelnut antibody towards walnut has also been observed previously (Holzhauser and Vieths, 1999; Kiening et al., 2005). The near taxonomic relationship of both species in the *Hamamelidae*

subclass might give an explanation. In case of the monocotyledon coconut, the cross-reactivity was more surprising although there is a clinical report about tree nut allergic patients who reacted to coconut protein (Teuber and Peterson, 1999), probably due to cross-binding of serum-IgE to walnut legumin-like proteins in the coconut endosperm tissue.

The key parameters and characteristics of the hazelnut TR-FIA were further determined in an in-house validation. The repeatability as measured by intra-assay precision was <9% and the reproducibility as measured by inter-assay precision was <20%. The method worked reliable in the low milligram per kilogram range and the performance was considered as satisfactory. The TR-FIA was robust with respect to minor changes; however, the choice of coating buffer was a sensitive determinant as tests during the method development showed. The DELFIA-assay buffer recommended by the manufacturer gave an at least 10-fold higher background noise, a reduced working range of the hazelnut protein standard curve and poor precision data. The use of PBS as coating buffer reduced the noise problem considerably, but caused spots of unexplainable high intensity randomly distributed on each microtiter plate.

The accuracy of the hazelnut TF-FIA as determined in recovery experiments was good with generally low variances (S.E.M. <11%), except for the lowest spiking level in cereals (S.E.M.=16%). In flour-containing foods like cookies and cereals, the recoveries were almost complete, whereas they were reduced in chocolate-containing foods, probably due to interfering chocolate constituents (Holzhauser et al., 2002). Adding 5% fish gelatin to the extraction buffer did not improve the results, but the addition of 1% dry milk increased the recoveries to  $\geq 100\%$ . However, the original extraction method was considered to be adequate for use in the food survey. Two conformational studies were performed: all food survey samples were re-analysed by rocket electrophoresis (RE) (Malmheden Yman et al., 1994). For samples containing hazelnut protein  $\geq 20$  mg/kg (LOD of the RE), a mean difference of  $\pm 30\%$  was calculated, which was considered as satisfactory, taking the accuracies of both methods into account. Secondly, 10 hazelnut protein control samples from an inter-laboratory validation study of two commercial hazelnut ELISA kits were measured in the hazelnut TR-FIA as an additional performance test for concentrations <2 mg/kg. The inter-kit deviations were 7–85% for the two commercial kits, and 0–62% and 0–72% for the TR-FIA in comparison with each kit, showing an acceptable congruity for these low protein levels. Nevertheless, the trueness of the TR-FIA could

not be determined as there is no certified hazelnut protein reference material available.

The sensitivity of the new TR-FIA with a LOD of 0.1 mg/kg hazelnut protein in food and a LOQ of 0.33 mg/kg seems sufficient to detect trace amounts in processed foods on levels presenting a risk for consumers with oral hazelnut allergy. Minimum provoking doses as determined by double-blind, placebo-controlled food challenges with raw, dried hazelnuts ranged from 1 to 100 mg hazelnut protein (Wensing et al., 2002). After ingestion of 6 mg hazelnut protein, allergic reactions would be experienced of 50% of the affected patients, stressing the need for sensitive analytical tools for consumer protection (Vieths, 2003).

A food survey with randomly-sampled Norwegian retail products labelled “may contain traces of nuts” was performed to scrutinise the application of this marking. Using the TR-FIA, traceable hazelnut concentrations were measured in 37% of all samples, i.e., in 32% of the cereals, 25% of the cookies, 69% of the milk chocolates and 50% of the dark chocolates. The hazelnut protein levels found varied considerably, they were low in cereals and cookies, and high in the chocolates. Milk chocolate was the most contaminated food product, corresponding to the results of surveys performed in Germany and Austria (Holzhauser and Vieths, 1999; Kiening et al., 2005). The high prevalence of hazelnut protein in commercial food items is probably a consequence of the practise of shared production lines of nut and nut-free products. The latter cannot be produced in facilities dealing with materials containing hazelnut because of the high risk of cross-contamination. Considering the imminent health risk for hazelnut-allergic patients, the labelling practise seems to be adequately handled by the food industry in the current situation.

In conclusion, a TR-FIA for the detection of hazelnut protein in foods was developed and validated. The  $\text{Eu}^{3+}$ -fluorescence used for detection contributes to reduce matrix interferences and to obtain a high sensitivity, so that the method is suited for trace analysis. The tracking of hazelnut as hidden allergen as demonstrated in a food survey can help to explain allergic incidents, and to support the food processing industry with the quality control of their production and products.

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