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ELISA detection of hazelnut proteins: effect of protein glycation in the presence or absence of wheat proteins

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Abstract

Hazelnuts are widely used in the food industry, especially confectionary foods. Nevertheless, these nuts contain several allergenic proteins which may be unexpectedly present as contaminants in various foods and may pose a serious threat to allergic consumers. The enzyme-linked immunosorbent assay (ELISA) is the preferred method to assess the level of hazelnut protein contamination. It is commonly used both by the food industry and enforcement agencies. Several ELISA kits are commercially available. However, protein detectability by ELISA may be affected by severe changes that proteins undergo during processing. The aim of this study was therefore to investigate the impact of processing on the ability to detect hazelnut protein by four commercial ELISA kits. Hazelnut proteins in the presence or absence of soluble wheat proteins were modified with glucose via the Maillard reaction. Changes in hazelnut proteins, such as the formation of protein bound carbonyls, losses of reactive lysine residues and free amino groups, and severe aggregation dramatically affected the hazelnut protein detection by the commercial kits. The observed impact was highly dependent on the type of ELISA kit used.

Keywords: Hazelnut allergy, ELISA, detectability, Maillard reaction, reactive lysine, protein bound carbonyls, aggregation

Introduction

Food allergy is an abnormal immunological response due to sensitization to a food component. Food allergies have become an important health problem, especially in industrialized countries, where they have been estimated to affect about 2% of the adult population and up to 8% of children (Monaci et al., 2006). Together with the hen's egg, cow's milk, fish, crustacean, peanut, soybean and wheat, tree nuts are typically associated with severe allergic reactions. Among the latter, hazelnut (*Corylus avellana*) allergy is becoming more important because of its increased incidence, which is related to the homology between the major birch pollen allergen *Bet v 1* and the allergens from hazelnut (Hansen et al., 2003). As a consequence, hazelnut allergies are predominantly secondary allergies. Hazelnut allergy can cause severe reactions and occurs mostly throughout the European population with a prevalence between 0.1 and 0.5% (Birmingham et al., 2005; deGroot et al., 1996). Moreover, numerous cases of hazelnut contamination of foods have been reported (Kiening et al., 2005; Pele Maria et al., 2007).

Given that the only effective treatment for hazelnut or other food allergies is the complete avoidance of the food containing the allergen, this represents an important food safety problem. Directive 2007/68/EC specifies a list of 14 allergic raw materials which must be labeled when present in the food as an ingredient (European Parliament and Council Directive 2007/68/EC, 2007). However, due to the growing complexity of food formulation and food processing, food may be unintentionally contaminated via allergen-containing ingredients or carry-over during processing. For this reason, accurate screening of the raw materials and the end products is essential. However, no "gold standard methods" exists for detection of food allergens. At present, the enzyme-linked immunosorbent assay (ELISA) is most frequently used in routine food analysis because it is relatively cheap, easy to perform and highly sensitive. ELISA methods are based on specific molecular interactions between

1
2
3 44 the antibodies used in the assay and the analyte, in this case proteins. During food processing,
4
5 however, proteins undergo changes such as aggregation, polymerization, degradation and
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7
8 46 random unfolding. Moreover, food proteins often interact with other food components during
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10 processing and can lead to additional modifications at the protein level. Therefore, the
11
12
13 48 antibody-protein interaction during the analytical procedure might be affected and potentially
14
15 lead to erroneous assay results (De Meulenaer et al., 2005).
16

17 50 Hazelnuts are frequently used in food products containing significant amounts of sugar
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19 (e.g. cookies, chocolate, breakfast cereals and, nut spreads). During thermal treatment and
20
21
22 52 storage, hazelnut proteins might be modified by the Maillard reaction, which occurs between
23
24 a reducing sugar and amino groups in proteins. The products formed as a result of Maillard
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26
27 54 reaction are significant in foods because they are responsible for flavor and color (Brands and
28
29 van Boekel, 2001). Abundant evidence exists for how thermal processing together with
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31
32 56 Maillard reaction can greatly influence the IgE binding activity of food allergens (Gruber et
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34 al., 2004; Gruber et al., 2005; Nakamura et al., 2005; Nakamura et al., 2008). In contrast, little
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36
37 58 information is available on how thermal processing and the Maillard reaction influence the
38
39 performance of commercially available ELISA kits for allergen detection. Such kits are often
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41
42 60 used in the food industry and official food agency laboratories to detect and quantify hidden
43
44 allergens in food. Recently, Scaravelli *et al.* demonstrated that thermal treatment of cookies
45
46
47 62 spiked with peanut caused a decline in the detection of *Ara h1* and *Ara h2* by two commercial
48
49 ELISA kits (Scaravelli et al., 2009).

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51 64 The aim of this study was to investigate whether the Maillard reaction in the presence or
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53 absence of soluble wheat proteins affects the detectability of hazelnut proteins in solution.
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56 66 This was done in 1% buffered protein solutions incubated with 6% glucose at 70°C for up to
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58 48 h. A model system with soluble wheat proteins was chosen, given that wheat is one of the
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68 68 bulk ingredients in cookies and breakfast cereals. First, the impact of the Maillard reaction on

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2
3 the hazelnut proteins was assessed by evaluating the losses of reactive lysine residues and of
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6 70 free amino groups, generation of protein bound carbonyls and changes in the SDS-PAGE
7
8 pattern. Additionally, the influence on the hazelnut protein detectability was assessed by
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10 72 analyzing the modified protein solutions by four commercially available ELISA kits:
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12 Veratox® for Hazelnut, Ridascreen® FAST Hazelnut, Hazelnut Residue and BioKits
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14
15 74 Hazelnut Assay were used for this purpose.
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Materials and methods

76 *Reagents and standards*

10 Soluble wheat proteins “Meripro 711” were kindly provided by Syral Belgium N.V.
11 (Aalst, Belgium). Chemicals and standards of analytical grade were purchased from Sigma-
12 78 (Aalst, Belgium). Chemicals and standards of analytical grade were purchased from Sigma-
13 Aldrich (Bornem, Belgium), VWR (Leuven, Belgium) and Acros Organics (Geel, Belgium).
14
15 Analytical grade solvents and technical grade hexane were obtained from Chem-Lab
16 80 (Zedelgem, Belgium). Gel and standards for SDS-PAGE came from Bio-Rad (Nazareth,
17 Belgium) while the Krypton protein stain was provided by Thermo Scientific (Aalst,
18 Belgium). The commercial ELISA kits used were Veratox® for hazelnut (Neogen, Lansing,
19 USA), Ridascreen® FAST Hazelnut (R-Biopharm, Darmstadt, Germany), BioKits Hazelnut
20 Assay (Tepnel, Flintshire, UK) and Hazelnut Residue (ELISA Systems, Queensland,
21 Australia).
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35 *Hazelnut protein extraction*

37 88 Hazelnuts from nine different brands were purchased in Belgian local supermarkets;
38 eight brands were virgin and one was roasted. A mixture of the hazelnuts was made by taking
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40 equal amounts of each kind. Hazelnuts were frozen with liquid nitrogen and ground in a two-
41 90
42 equal amounts of each kind. Hazelnuts were frozen with liquid nitrogen and ground in a two-
43 step process, first with a blender (Moulinex, France) then with an Ultra Turrax t25 (IKA,
44
45 Wilmington, US).
46
47 92
48

49 The in-house prepared hazelnut protein extracts were obtained following Yeung and
50 Collin’s protocol (Yeung and Collins, 1996). In summary, 100 g of ground nuts were defatted
51 94
52 four times using 1 L technical hexane each time. After allowing the solvent to evaporate
53
54 overnight at ambient temperature, 10 g of the defatted powder was mixed with 100 mL of
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56 96
57 extraction buffer and mixed at 4°C over night. Protein extraction buffer consisted of 1 M
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59 NaCl, 1.47 mM KH₂PO₄, 2.7 mM KCl and 10 mM Na₂HPO₄. Sodium azide 0.2 g L⁻¹ was
60 98

1
2
3 added to the extraction buffer to avoid microbial growth. Further, a mixture of protease
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6 100 inhibitors (2 mM ethylenediaminetetraacetic acid (EDTA), 5 mM sodium diethyl-
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8 dithiocarbamate (DIECA), 0.5 mM benzamidine hydrochloride (BAHC) and 0.2 mM
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10 102 phenylmethylsulfonyl fluoride (PMSF)) was added to the extraction buffer to increase the
11
12 stability of the protein isolate during extraction and storage (Bjorksten et al., 1980). The
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14
15 104 protein mixture was further centrifuged for 1h at 4°C and 20000 g to separate the lees.
16
17 Subsequently, the clear supernatant was dialyzed (Spectra/Por 3 membrane tubing, MWCO
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19 106 3.5 kDa) using the extraction buffer with the protease inhibitors containing only 0.137 M
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21 NaCl, for 48h at 4°C and then filtered through a 0.45 µm membrane filter (Puradisc FP 30,
22
23
24 108 Novolab, Belgium). Finally, the protein solution was lyophilized and stored at -20°C until
25
26 further use. This extract was used as the reference extract.
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30 110 *Maillard reaction in the absence of soluble wheat proteins*

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32
33 Hazelnut protein extracts were dialyzed in 0.1 M phosphate buffer pH 7.4 (Spectra/Por
34
35 112 3 membrane tubing, MWCO 3.5 kDa) before treatment to remove the nitrogen-containing
36
37 protease inhibitors. All reaction systems were prepared in the same phosphate buffer (pH 7.4)
38
39
40 114 by mixing 1% (w/v) hazelnut proteins with 6% (w/v) glucose. The reaction systems in closed
41
42 falcon tubes were incubated in a water bath at 70°C for up to 48 h. A glucose-free reaction
43
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45 116 system was used as a control. Samples were retrieved from the water bath and placed in an ice
46
47 bath to stop the reaction at specific times. Further, the modified protein solutions were
48
49
50 118 immediately used for the determination of reactive lysine residues, protein bound carbonyls,
51
52 free thiol and free amino groups. Aliquots for SDS-PAGE were frozen at -20°C until further
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54 120 use.
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Maillard reaction in the presence of soluble wheat proteins

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6 122 Two types of glucose-containing model systems were prepared. One model system was
7
8 obtained by mixing 0.25% (w/v) of hazelnut proteins with 0.75% (w/v) of wheat proteins and
9
10 124 6% (w/v) of glucose. Another model system was obtained by mixing 0.5% (w/v) hazelnut
11
12 proteins with 0.5% (w/v) wheat proteins and 6% (w/v) glucose. Respective model systems
13
14
15 126 without glucose were prepared as control. These systems were then treated similarly as the
16
17 model systems containing no soluble wheat proteins.
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Assessment of the Maillard reaction

Determination of the protein bound carbonyls

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21 128
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27 130 Protein bound carbonyls were measured after converting them to the corresponding 2,
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29 4- dinitrophenylhydrazones. Briefly, 300 μL protein solution was incubated with 400 μL 2,4-
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31 132 dinitrophenylhydrazine (DNPH) 10 mM in 2 M HCl. Further, the proteins were precipitated
32
33 with 10% trichloroacetic acid (final concentration) and thoroughly washed with 1 mL
34
35 ethanol/ethyl acetate (1:1) after derivatization; hence any unreacted DNPH or DNPH bound to
36
37 134 any other components than protein, was removed. Finally, the pellet was redissolved in 6 M
38
39 urea and absorbance was measured at 370 nm using a Bio-Rad Benchmark Plus microplate
40
41 136 spectrophotometer (Nazareth, Belgium). The carbonyl content was calculated using a molar
42
43 absorption coefficient of $22000 \text{ M}^{-1} \text{ cm}^{-1}$ and the results were expressed in nmol bound
44
45 138 carbonyls/mg protein.
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Loss of reactive lysine residues

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52 140
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54 The loss of reactive lysine residues was monitored using derivatization with *ortho*-
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56 142 phthaldialdehyde (OPA) in the presence of 2-mercaptoethanol yielding a fluorescent product
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58 with a maximum excitation wavelength of 340 nm and emission of 450 nm (Ferrer et al.,
59
60 144 2003) and measured by a Spectramax Gemini XPS fluorimeter from Molecular Devices

(Brussels, Belgium). A standard of β -casein was used to prepare a calibration curve and samples were pre-treated and analyzed using a fluorimeter as described previously (Morales et al., 1996).

148 *Free amino groups determination*

The modification of free amino groups was determined using derivatization with 2, 4, 6-trinitrobenzene sulfonic acid (TNBS) as previously described (Fields, 1972). Briefly, 0.5 mL of protein solution (containing between 0.05 – 0.25 mg proteins) was incubated with 0.5 mL of 0.1 M tetraborate buffer prepared in 0.1 M NaOH. Next, 20 μ L of 0.5% TNBS solution was added to the proteins and the reaction mixture was incubated for exactly 10 minutes at room temperature. To stop the reaction, 2 mL of stop solution (1.5 mL of 0.1 M $\text{Na}_2\text{S}_2\text{O}_3$ with 98.5 mL of 0.1 M NaH_2PO_4) were added. The absorbance of the resulting mixture was read at 420 nm using a Cary 50 UV-Vis Spectrophotometer from Varian (Sint-Katelijne-Waver, Belgium). The amount of free amino groups was calculated using a molar absorption coefficient of $22000 \text{ M}^{-1} \text{ cm}^{-1}$ and results were expressed in nmol free amino groups/mg protein.

160 *SDS-polyacrylamide gel electrophoresis (SDS-PAGE)*

Protein cross-linking or fragmentation was analyzed by SDS-PAGE under reducing conditions. A 4% acrylamide stacking gel and a 10% acrylamide resolving gel were used for protein separation. An aliquot of 10 μ L sample containing 6 μ g of protein was added to 10 μ L Laemmli sample buffer containing β -mercaptoethanol and heated at 95°C for 5 min. The samples were further loaded on the gel along with a Precision plus protein unstained molecular weight marker (Bio Rad, Nazareth, Belgium). Krypton fluorescence staining was used to visualize the bands. The gels were read using the Molecular Imager PharosFX Plus

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2
3 168 from Bio Rad (Nazareth, Belgium) and the respective molecular weights of the hazelnut
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5 proteins were recorded.
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7

8
9 170 *Protein determination*

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11 Protein determination was performed by determining the nitrogen content according to
12
13 172 the Kjeldahl procedure (AOAC Official Method 981.10, 1981). A factor of 5.41 was used to
14
15 convert nitrogen to total hazelnut protein, and 6.25 to convert to total protein content for the
16
17 hazelnut wheat protein mixtures. The non-protein nitrogen (NPN) fraction was determined in
18
19 174 the supernatant after a previous protein precipitation with 15% TCA (final concentration).
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23
24 176 *ELISA assays*

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27 Samples of the above-described model systems were analyzed using four different
28
29 178 commercial ELISA kits: Veratox® for hazelnut (Neogen), Ridascreen® FAST Hazelnut (R-
30
31 Biopharm), BioKits Hazelnut Assay (Tepnel) and Hazelnut Residue (ELISA Systems). The
32
33 four kits were applied to the different model systems. These model systems included (1) 1%
34
35 hazelnut proteins, (2) 1% hazelnut proteins with 6% glucose, (3) 0.5% hazelnut + 0.5%
36
37 soluble wheat proteins, (4) 0.25% hazelnut + 0.75% soluble wheat proteins, (5) 0.5%
38
39 182 hazelnut + 0.5% soluble wheat proteins and 6% glucose, (6) 0.25% hazelnut + 0.75% soluble
40
41 wheat proteins and 6% glucose. Of the different model systems, samples retrieved after 0, 24
42
43 184 and 48 h incubation at 70°C were analyzed. The untreated sample (= 0 h of thermal treatment)
44
45 of the glucose-free model system (1) containing only hazelnut proteins is referred to as the
46
47 186 reference sample. Before the analysis, dilution series were made of the different samples in
48
49 the extraction buffer or dilution solution of the respective kits. The actual concentrations are
50
51 the concentrations of hazelnut proteins based on the absolute protein determination using the
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53 188 Kjeldahl procedure and do not include the wheat proteins that were added in some of the
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55 190 model systems. The dilution series of the samples were analyzed in duplicate in the different
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3 192 kits according to the manufacturers' instructions. Absorbance measurements were made in a
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5
6 microtiter plate reader (Multiskan MCC/340, Titertek, Huntsville, Alabama). Each kit was
7
8 194 calibrated using the respective standard protein solutions included in each kit. For fitting the
9
10 absorbance values to the protein concentration of the standard solutions, a four-parameter-
11
12 logistic dose-response curve was used with equation 2.1 below. This equation represents a
13 196
14 standard way to express immunoassay data, where a is the maximum dose (protein
15
16 concentration) of the curve, b is the minimum concentration, c is the E_{50} -value (concentration
17 198
18 at half-maximal saturation), d is the slope of the curve and $conc$ is the concentration of the
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20 standard or sample (Englebienne, 2000).
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22 200
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$$24$$

$$25$$

$$26$$

$$27 \text{ Absorbance} = \left[\frac{(b-a)}{\left(1 + \frac{conc}{c}\right)^d} \right] + a \quad (2.1)$$

$$28$$

$$29$$

$$30$$

31 202 For the Ridascreen® FAST Hazelnut data were also fitted according to a cubic spline function
32
33 as described in the manufacturer's instructions. In case of the Veratox for Hazelnut the kit's
34
35 procedure recommended fitting the data to log-logit function, which is a linearization of the
36 204
37 four-parameter logistic function. For the Hazelnut Residue and the BioKits Hazelnut Assay
38
39 no suggestion was made with respect to curve fitting. Only the data obtained within the range
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41 206
42 of the respective standard curves were considered in the results. Outlying data are indicated
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44
45 208 by an asterisk (*) in the charts.
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210 Results

211 *Progress of the Maillard reaction*

212 The interaction of reducing sugars or dicarbonyl compounds derived from sugars may
213 lead to the formation of protein-bound carbonyls (Stadtman and Levine, 2003). Therefore, the
214 amount of protein bound carbonyls was used as an indicator of the Maillard reaction. A time-
215 dependent increase of protein bound carbonyls was observed in all model systems used
216 (**Figure 1**). In the pure hazelnut protein samples, an almost seven-fold increase was observed
217 after incubation for 48 h in the presence of glucose while incubation in the absence of glucose
218 led to only a slight increase in protein carbonyls (about a three-fold increase). Incubation of
219 hazelnut proteins in the presence of wheat proteins and glucose similarly led to an increase in
220 the measured protein bound carbonyls, but less than the similar model systems in which no
221 wheat proteins were present.

222 When incubating hazelnut proteins with glucose a gradual decrease of the reactive
223 lysine residues was observed as incubation time increased. This led to a 64% loss after 48 h of
224 incubation. (**Figure 2**). In contrast, incubation in the absence of glucose did not affect the
225 reactive lysine content of the hazelnut protein, even after 48 h of incubation. The reaction
226 systems containing 0.5% and 0.75% wheat proteins contained slightly lower amounts of
227 reactive lysine residues as compared with the model system containing no wheat proteins,
228 which is in line with the lysine deficiency of wheat proteins. Upon incubation, 60 to 70%
229 losses of reactive lysine residues during the first 24 h were observed, while further incubation
230 did not further affect the reactive lysine content.

231 **Figure 3** shows the SDS-PAGE pattern of the hazelnut protein incubated with glucose
232 in the presence or absence of soluble wheat proteins. The SDS-PAGE pattern of the native

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2
3 hazelnut proteins is characterized by the presence of proteins in the molecular weight range of
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5 234 10 to 80 kDa. The major hazelnut allergens detected were the *Bet v 1* related *Cor a 1* and the
6
7 oleosin *Cor a 12*, both with a molecular weight of 17 kDa; *Cor a 9* - a 11S globulin with
8
9 acidic (35-40 kDa) and basic (~20kDa) subunits, and *Cor a 11* which represents a 48 kDa
10 236 glycoprotein. Further, *Cor a 10*, a luminal binding protein of 73 kDa was detected along with
11
12 the profilin *Cor a 2* of 14 kDa (Robotham et al., 2009). When soluble wheat proteins were
13
14 present in the model systems the intensity of the main hazelnut proteins bands became weaker
15 238
16
17 due to the lower amounts of hazelnut present. Wheat proteins used in the model systems were
18
19 partially hydrolyzed resulting in a lack of clearly visualized bands.
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24 242 Upon incubation of hazelnut proteins with glucose, high molecular weight protein
25
26 clusters appear as a smear in the SDS-PAGE pattern. Moreover, prominent aggregate
27
28 formation occurred, which is manifested by their presence at the boundary of the resolving
29 244
30
31 and stacking gel, as well as in the stacking gel. The high-molecular mass aggregates on top of
32
33 the gel could not migrate into the stacking gel. They probably have molecular masses larger
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35
36 than 300 kDa. Upon addition of the soluble wheat proteins, it was obvious that the formation
37
38 of these high molecular weight complexes was partially inhibited in a wheat protein
39 248
40
41 concentration dependent manner. Nevertheless, after 48 h of incubation, smearing and
42
43 formation of high molecular weight aggregates were still visible.
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47 ***Detection with commercial ELISA kits***

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49 252 The concentration of the standards included in three of the four kits tested (Veratox®
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51 for hazelnut, Ridascreen® FAST Hazelnut and BioKits Hazelnut Assay) is expressed as a
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53 particular amount of *hazelnut*. However, the samples of the different prepared model systems
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55
56 have a known or actual *hazelnut protein* concentration. To be able to compare the actual
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58 concentration of the samples that were analyzed with the measured concentration derived
59 256
60
61 from the calibration curve obtained with the kit's standards, the concentration of the standards

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2
3 258 was converted to the same dimension of that from the model system samples. This was done
4
5 according to the formula below (3.1), taking into consideration the protein content of the
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7
8 260 hazelnut standards as indicated in the manual of the kit or, if not indicated, a protein content
9
10 of 12% was used (Belser et al., 1999).
11

12 262

$$15 \text{ concentration hazelnut} \times \text{protein content (\%)} = \text{concentration hazelnut protein} \quad (3.1)$$

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17 264

19
20 The hazelnut or hazelnut protein concentration of the kit's standards is expressed in
21
22 266 parts per million (ppm) which equals ng analyte/mg food product. However, as the
23
24 concentration of the hazelnut proteins in the model systems is expressed in ng mL⁻¹, the
25
26
27 268 concentrations of the kit's standards were converted according to formula 3.2, considering the
28
29 weight of the sample (mg) extracted by a particular volume of extraction buffer (mL)
30
31 according to the respective manufacturers' instructions.
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$$38 \text{ ppm} = \frac{\text{ng analyte}}{\text{mg food}} \times \frac{\text{weight of extracted sample (mg)}}{\text{volume of extraction buffer used (ml)}} = \frac{\text{ng analyte}}{\text{ml solution}} \quad (3.2)$$

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42 274 For the evaluation of the kits' performance, a dilution series of the hazelnut protein
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44 solutions obtained from the six different model systems were applied in the appropriate
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46 concentration range of each kit. The obtained absorbance values were converted to a
47 276 measured hazelnut protein concentration, which was compared with the actual protein
48
49 concentration presents in each diluted solution (Figure 4).
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52 278
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54 55 56 *Veratox for hazelnut*

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58 280 For the "Veratox® for hazelnut" kit, calibration curves established with the standard
59
60 hazelnut solutions provided in the kit, were fitted to the four-parameter-logistic function. The

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2
3 282 log-logit fit, according to the manufacturer's instructions, is a linearization of the sigmoid
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5 shape of the four-parameter logistic function. Consequently, both fittings return the same
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7
8 284 concentration for a given absorbance value. After conversion of the concentrations according
9
10 to formulas 3.1 and 3.2, the standards covered a range from zero to 120 ng mL⁻¹ hazelnut
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12 286 protein. The actual hazelnut protein concentration present in the reference sample and its
13
14 dilutions was dramatically underestimated - on average by 94% (**Figure 4A**). Upon heating
15
16
17 288 the reference sample for 24 h at 70°C without glucose, the discrepancy increased between the
18
19 actual hazelnut protein concentration present and the protein concentration measured by the
20
21
22 290 kit. Only up to 4% of the actual hazelnut protein present could be detected (data not shown).
23
24 When the heat treatment up to 48 h the detectability decreased down to about 3.3% on
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26
27 292 average.

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32 294 Once soluble wheat proteins (0.75%) were added to the reference hazelnut protein
33
34 sample (0.25%), an underestimation was again observed. Only 12.6% of the actual hazelnut
35
36 296 protein concentration by the kit. This was only marginally less dramatic compared to the
37
38 reference protein samples to which no wheat proteins were added. Remarkably, if a lower
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40 amount of wheat proteins was present (0.5% hazelnut + 0.5% soluble wheat proteins) even
41
42 298 less of the actual hazelnut protein concentration was observed (9.6% detected, not shown).
43
44 Upon heating the mixture of hazelnut and soluble wheat protein solutions a further decrease in
45
46 300 the measured hazelnut protein concentration was observed, although the decrease was less
47
48 pronounced compared to the similarly treated samples in the absence of soluble wheat
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50
51 302 proteins (17% compared to 45% loss in signal compared to the native reference sample in the
52
53 absence and presence of soluble wheat proteins, respectively).

54
55 304 Upon addition of glucose during heating, the discrepancy between the actual and
56
57 measured hazelnut protein concentration increased dramatically, both in the absence and
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3 306 presence of soluble wheat proteins (2% or less of the actual hazelnut protein concentration
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5 was measured).
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9 308 *Ridascreen® FAST Hazelnut*

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11 The values obtained for the standards using the “Ridascreen® FAST Hazelnut” kit were
12
13 fitted to the four-parameter-logistic and a cubic spline function. After conversion according to
14 310
15 the formulas given above, the standards ranged from zero up to 90 ng mL⁻¹ hazelnut protein.
16
17 After analysis of a dilution series of the reference hazelnut protein sample with the
18 312
19 Ridascreen® FAST Hazelnut only the data for the samples actually containing less than 50 ng
20
21 mL⁻¹ hazelnut protein could be considered, as the samples with a higher concentration
22
23 314
24 returned absorbance values outside the range of the kits standard curve.
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27
28 316 The measured concentrations of the reference sample were underestimated by about
29
30 17% using the logistic standard curve, except for the lowest concentration (5 ng mL⁻¹), which
31
32 was underestimated by 49% (**Figure 4B**). With the cubic spline fit the calculated
33 318
34 concentrations were about 10% higher than with the four-parameter logistic function.
35
36 However, using the former fitting, no calculations could be made for samples with an
37 320
38 absorbance value between zero and that of the first standard. Heat-treatment of the reference
39
40 sample at 70°C led to a limited loss in detectability after 48 h (about 13%).
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42 322

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44 When soluble wheat proteins were added (0.75%) to the reference hazelnut protein
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46 324 sample (0.25%) the protein concentration was either underestimated by 12% (40 ng mL⁻¹) or
47
48 overestimated by 15% (20 ng mL⁻¹). For the lowest concentration (4 ng mL⁻¹) the obtained
49
50 signal resulted in a calculated concentration equal to zero. In the presence of a lower amount
51 326
52 of soluble wheat proteins (0.5% hazelnut protein + 0.5% soluble wheat proteins) the samples
53
54 were underestimated by 80% on average (data not shown). Heating alone of the mixture of
55 328
56 hazelnut and soluble wheat proteins, led to a loss in detectability (signal reduced on average
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58 by 30% after 24 h with an additional loss of 15% after 48 h incubation as compared to the
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3 native samples). This loss in detectability was more pronounced as compared with the
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5
6 332 similarly treated reference hazelnut protein solution in the absence of wheat proteins (13%
7
8 reduction). Addition of glucose during heating led to a greater discrepancy between the actual
9
10 334 and measured concentrations. In the presence of wheat proteins the signal further decreased
11
12 further by about 30%. Interestingly, in the absence of wheat proteins an increased
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14
15 336 detectability was observed after heating for 48 h.

18 *Hazelnut Residue*

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21 338 The calibration curves obtained with the standards of the “Hazelnut Residue” kit were
22
23 fitted to the four-parameter logistic function. The converted concentrations of the standards
24
25
26 340 ranged from zero to 500 ng mL⁻¹. The actual hazelnut protein concentration of a dilution
27
28 series of the reference sample was underestimated on average by 45% (**Figure 4C**). Overall,
29
30 342 small fluctuations in detection were observed upon heating the samples at 70°C up to 48h in
31
32 the absence of glucose as compared with the reference sample. Addition of soluble wheat
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34
35 344 proteins (0.25% hazelnut + 0.75% soluble wheat proteins) resulted in increased detection,
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37 leading to an overestimation by an average of 34% for the samples considered. It should be
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39
40 346 noted that the overestimation occurred especially in the more diluted sample solutions. In
41
42 addition, due to the overestimation in the presence of soluble wheat proteins, some samples
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44
45 348 were out of range (800 and 400 ng mL⁻¹). When a lower amount of wheat proteins was added
46
47 (0.5% hazelnut + 0.5% wheat proteins) the considered samples were now underestimated by
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49
50 350 24% on average (data not shown). Upon heating the samples containing hazelnut and soluble
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52 wheat proteins alone, the measured hazelnut protein concentration decreased by 45% on
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54 352 average. Upon addition of glucose to the reference sample during heating, the discrepancy
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56 between the actual and measured hazelnut protein concentration increased further (44% loss
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59 354 in signal compared to the sample heated in the absence of glucose), which was even more
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pronounced in the presence of soluble wheat proteins (63% loss in signal).

356 *Biokits Hazelnut Assay*

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6 After conversion according to formula 3.1 and 3.2, the standards of the “Biokits Hazelnut
7
8 358 Assay” covered a range from zero up to 192 ng mL⁻¹ hazelnut protein and the corresponding
9
10 absorbencies were fitted according to the four-parameter-logistic function. The actual
11
12 concentrations of the considered dilutions of the reference sample were on average
13 360 overestimated by 27% (**Figure 4D**). Upon heating the reference sample for 24 h at 70°C
14
15 without glucose, the measured concentration of the samples decreased by 7% (data not
16
17 362 shown) with an additional loss in signal of 20% when the heating was extended to 48 h. When
18
19 adding soluble wheat proteins (0.75%) to the reference sample (0.25% hazelnut proteins) the
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21 actual concentration was overestimated by about 50% on average, especially at the lower
22
23 364 concentration range, while this overestimation became only 20% when a lower amount of
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25 wheat proteins (0.5% hazelnut and 0.5% wheat proteins) was added (not shown). Upon
26
27 366 heating in the absence of glucose the overestimation became an underestimation (14% on
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29 average) When glucose was included during the heat-treatment of the samples, the
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31 370 discrepancy between the actual and the measured hazelnut protein concentrations further
32
33 increased, both in the presence and the absence of wheat proteins. This observation was also
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35 372 more pronounced in the presence of soluble wheat proteins (48% loss in signal compared to
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37 the sample heat in the absence of glucose).
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4 374 **Discussion**
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7 A comparison of the kits manuals revealed interesting differences that affected the
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9 376 interpretation of the analytical results. Some of the kits express the results as amount of
10 hazelnut present, while others express the results as amount of hazelnut protein present. Given
11
12 378 that the allergen in question is hazelnut proteins and since the protein content of hazelnut
13 shows some variability, it seems more appropriate to express the results as the content of
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15 380 hazelnut protein. Another difference observed between Ridascreen Fast Hazelnut and the
16 other kits concerned the fitting of the calibration curve to a particular equation. The latter kit
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18 382 suggested using the cubic spline function. However, the equilibrium reactions involved in a
19 immunoassay make it more appropriate to use the four logistic function, or its linearized log-
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21 384 logit equivalent. Moreover, the use of the alternative cubic spline function does not allow
22 quantification of signals between zero and the first point of the calibration curve.
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33 386 Use of the reference extract (obtained from a mixture of various raw and one roasted
34 hazelnut lots) in the various kits yielded remarkable differences in their performance. The
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36 388 Ridascreen Fast Hazelnut kit and the Biokits Hazelnut assay proved to be the most accurate,
37 while the Veratox for Hazelnut kit showed results that underestimated by one order of
38
39 390 magnitude the actual hazelnut protein present in the reference extract. Interestingly, the
40 accuracy of some kits depended somewhat upon the actual hazelnut protein concentration
41
42 392 present in the solutions applied. Two possible causes for the differences observed between the
43 actual hazelnut protein concentration present in the applied reference solutions and the
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45 394 concentration obtained by the respective kits could be suggested. One, the antibodies used in
46 the respective kits could have been raised towards pooled hazelnut proteins extracted from
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48 396 different hazelnut varieties than those used for our reference extract. Two, the ratio's of the
49 different varieties in our reference mixture could also have differed. Indeed, De Meulenaer et
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60 398 al. (2005) observed a difference in the response of antibodies raised towards the protein

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3 extracted from Runner peanuts and those extracted from various other peanut varieties. The
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6 400 kits' manufacturers could also have used a protein extract from roasted hazelnuts instead. Our
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8 reference protein extract was based upon a mixture of 9 different hazelnut samples, of which
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10 402 only one was roasted. Indeed, as can be observed from various evaluated chemical parameters
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12 linked to the Maillard reaction, it was obvious that our reference extract did not contain
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15 404 severely modified proteins.

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17 Heating the reference hazelnut protein solution in the absence of glucose, which
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19 406 restricted the Maillard reaction as confirmed from the Maillard reaction parameters monitored
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21 (Figures 1 – 4), further decreased the accuracy for the Veratox for Hazelnut kit and the
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24 408 Biokits Hazelnut assay. In the case of the former assay, the loss in accuracy became quite
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26 dramatic, with less than 10% of the hazelnut protein present being detected. Some of the
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28 hazelnut proteins seem to be heat labile and denature upon incubation at 70°C. Because of
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30 410 this, the kits were able to detect less as compared with the native proteins. For the other kits
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33 412 evaluated only minor losses in accuracy could be noted, or in the case of the Hazelnut
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35 Residue kit, even a slightly increased accuracy.

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38 414 Upon heating the reference protein solution in the presence of glucose typical chemical
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40 indicators used to assess the impact of the reaction showed a progressive increase as a
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43 416 function of incubation time (Figure 1 and 2). These changes also clearly indicated that severe
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45 modifications on the molecular level of the hazelnut proteins occurred. Moreover, these
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48 418 modifications gave rise to a dramatic loss in the accuracy in three of the four kits evaluated
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50 (only the Ridascreen Fast Hazelnut assay remained accurate). For the latter assay, even a
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53 420 better binding occurred between the antibodies and the Maillard modified proteins compared
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55 to the proteins in the non-heated reference extract, most probably indicating that the
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58 422 antibodies used in that assay alone were developed towards modified hazelnut proteins.

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6 424 reference hazelnut protein solution in the presence of glucose, severe protein cross-linking
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8 occurred resulting in formation of high molecular weight aggregate formation. Protein cross-
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10 426 linking in the Maillard reaction typically originates because reactive nucleophilic groups such
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12 as lysine (**Figure 2**) react with the reactive carbonyl intermediates originating from the
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14 degraded reducing sugars. Since these reactive groups are present in several proteins, multiple
15 428 proteins are linked to each other resulting in the formation of large molecular complexes. It is
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17 thus important to note that allergen quantification is typically performed in foods in which the
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19 considered allergen is present as a minor compound next to major ingredients, including bulk
20 430 proteins. As a consequence, the traces of hazelnut proteins will primarily cross-link with these
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22 bulk proteins instead of cross-linking with the traces of other hazelnut proteins present.
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24 432 Therefore, the hazelnut proteins surface may become covered with bulk proteins, making the
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26 receptor-based detection using antibodies even more vulnerable to inaccuracy. In order to
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28 evaluate this hypothesis, mixtures of the reference protein with soluble wheat proteins were
29 434 heated in the presence of glucose. Soluble wheat proteins were taken as a case example in
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31 order to avoid solubility problems of the modified proteins as a result of progressive Maillard
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33 reaction. Since the soluble wheat protein consisted of a partially hydrolyzed mixture of wheat
34 436 proteins, considerably less protein aggregation occurred. This can be explained by the lower
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36 chance of retrieving multiple reactive nucleophilic groups in smaller protein fragments than
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38 in intact proteins. Nevertheless, cross-linking between hazelnut proteins and soluble wheat
39 438 proteins did occur. Cross-linking proved to have dramatic consequences on the accuracy of all
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41 kits evaluated, which could be easily explained by the molecular modification induced on
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43 proteins and consecutively on the receptor-based principle of immune assays.
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46 In view of the results obtained, it is obvious that extreme care should be taken by using
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48 receptor-based assays, such as ELISA kits for allergen detection. None of the evaluated
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3 448 ELISA kits produced accurate analytical results, even when non - treated reference protein
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5 solution was used. Even if the observed underestimation with the reference proteins solution
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8 450 used in our study is due to the use of a hazelnut pool which is not similar to the ones used to
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10 raise antibodies in these kits, it should be noted that these kits are used worldwide to asses
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12 452 contamination levels of hazelnuts of different origins, and modified to different degrees. As a
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14 consequence, care should be taken when using these kits for quantitative analysis, especially
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17 454 in view of enforcement of for instance EC Directive 2007/68/EU. Special attention should be
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19 also drawn when qualitatively interpreting the analytical data obtained. It was striking to
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22 456 observe that the accuracy of the kits evaluated not only depended upon the chemical
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24 modifications induced in the reference protein extract, but also depended upon the actual
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27 458 protein concentration present in the evaluated solutions. For some of the kits evaluated, a
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29 considerable lack of accuracy was observed, especially at low hazelnut protein concentrations.
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32 460 Given that kits for allergen detection are used for trace analysis, one would expect that the
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34 concentration of the target proteins in the food extract to be low. The above results clearly
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37 462 show that both false positive or false negative results from the commercial ELISA kits are
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39 likely to occur. False negative results present a potentially fatal risk for allergic consumers,
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42 464 while false positive results may harm the food industry through unnecessary and costly
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44 product withdrawal. Finally, it should be realized that the final outcome of any analytical
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47 466 method is influenced by the extractability of the analyte from the food matrix. In this
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49 perspective it is important to note that protein solubility is typically affected by progressive
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52 468 Maillard reactions. Considering the results obtained in this study, it can be concluded that,
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54 apart from issue related to the extractability of the proteins, also chemical modifications
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57 470 occurring on protein level as a result of the Maillard reaction may seriously affect the
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59 outcome of receptor based analytical methods, such as ELISA's.
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Figure captions

554 **Figure 1** Formation of protein carbonyls in hazelnut protein in the presence or absence of
soluble wheat proteins due to incubation with 6% glucose. The data points represent mean
556 values \pm SD of four independent determinations. \diamond 1% hazelnut proteins with 6% glucose; \square
0.5% hazelnut with 0.5% soluble wheat proteins and 6% glucose; Δ 0.25% hazelnut and
558 0.75% soluble wheat proteins and 6% glucose; \circ 1% hazelnut proteins

Figure 2 Loss of reactive lysine due to incubation of hazelnut proteins in the presence or in
560 the absence of soluble wheat proteins with 6% glucose, data points represent mean values
 \pm SD of four independent determinations. \diamond 1% hazelnut proteins with 6% glucose; \square 0.5%
562 hazelnut with 0.5% soluble wheat proteins and 6% glucose; Δ 0.25% hazelnut and 0.75%
soluble wheat proteins and 6% glucose; \circ 1% hazelnut proteins

564 **Figure 3** SDS-PAGE pattern of the hazelnut proteins incubated in the presence or absence of
wheat proteins with glucose: 1% hazelnut proteins and 6% glucose non treated (lane 1),
566 incubated for 24 h (lane 2) and 48 h (lane 3). 0,5% hazelnut proteins with 0,5% wheat
proteins and 6% glucose non treated (lane 4), incubated for 24 h (lane 5) and for 48 h (lane 6).
568 0,25% hazelnut proteins with 0,75% wheat proteins and 6% glucose non treated (lane 7),
incubated for 24 h (lane 8) and 48 h (lane 9). Molecular weight marker (lane 10)

570 **Figure 4** Ratio of measured over actual hazelnut protein concentration (Y-axis) in different
ELISA kits after *in duplo* analysis of a dilution series (X-axis) of a reference hazelnut protein
572 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
574 proteins. (* = absorbance value of sample outside the range of the standard curve)

■ - reference sample, ■ - reference heated for 48 h, ■ - reference heated for 48 h in the
576 presence of glucose, ■ - 0.25% hazelnut proteins and 0.75% soluble wheat proteins, ■ -

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3 0.25% hazelnut proteins and 0.75% soluble wheat proteins heated for 48 h, □ - 0.25%

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5 578 hazelnut proteins and 0.75% soluble wheat proteins heated for 48 h in the presence of glucose
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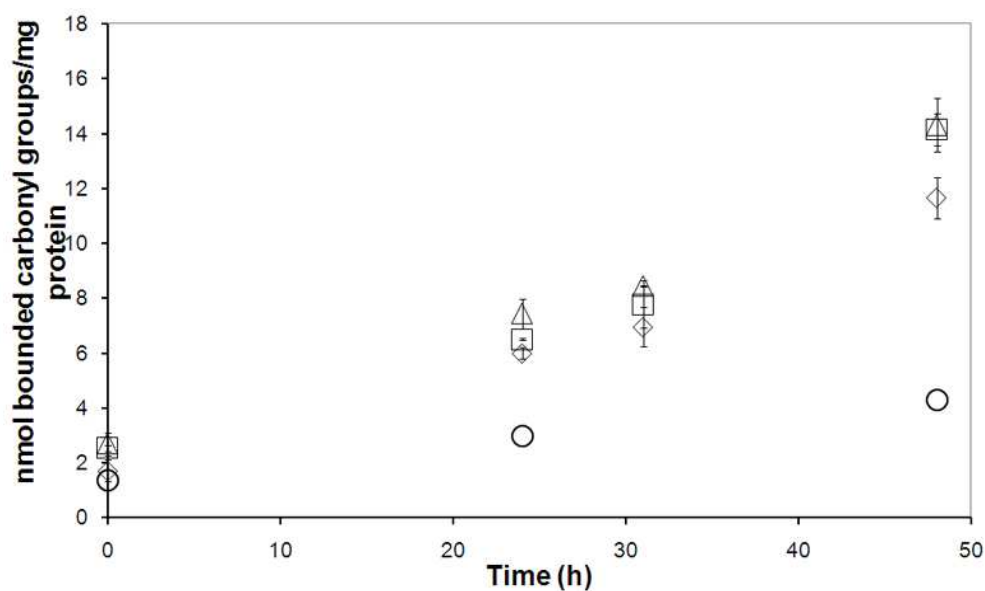


Figure 1 Formation of protein carbonyls in hazelnut protein in the presence or absence of soluble wheat proteins due to incubation with 6% glucose. The data points represent mean values \pm SD of four independent determinations. \diamond 1% hazelnut proteins with 6% glucose; \square 0.5% hazelnut with 0.5% soluble wheat proteins and 6% glucose; \triangle 0.25% hazelnut and 0.75% soluble wheat proteins and 6% glucose; \circ 1% hazelnut proteins

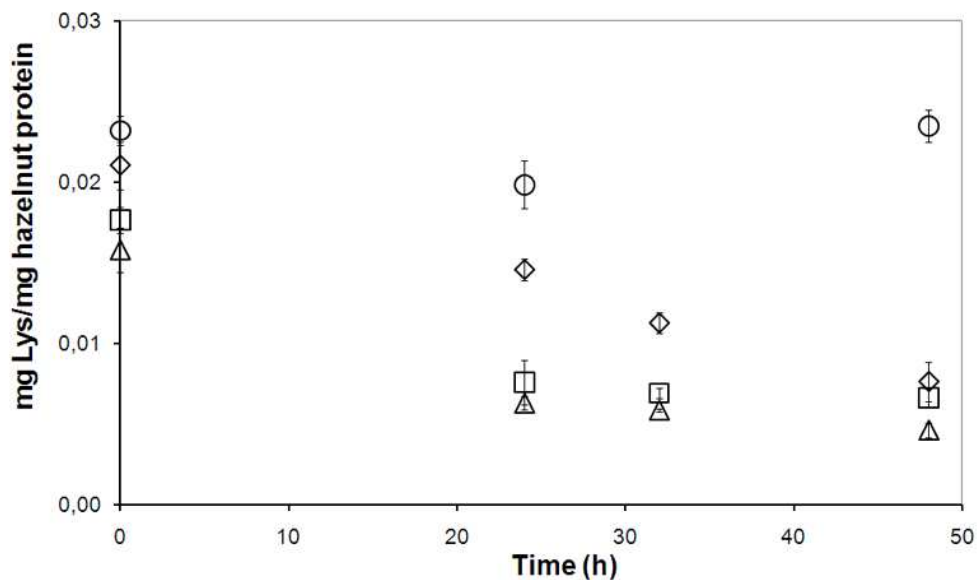


Figure 2 Loss of reactive lysine due to incubation of hazelnut proteins in the presence or in the absence of soluble wheat proteins with 6% glucose, data points represent mean values \pm SD of four independent determinations. \diamond 1% hazelnut proteins with 6% glucose; \square 0.5% hazelnut with 0.5% soluble wheat proteins and 6% glucose; \triangle 0.25% hazelnut and 0.75% soluble wheat proteins and 6% glucose; \circ 1% hazelnut proteins

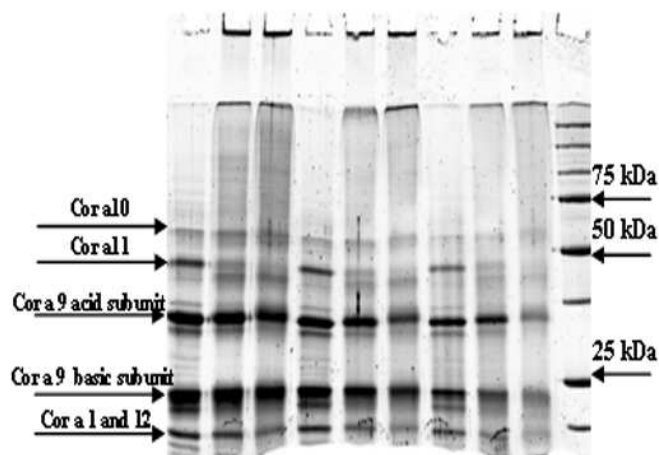


Figure 3 SDS-PAGE pattern of the hazelnut proteins incubated in the presence or absence of wheat proteins with glucose: 1% hazelnut proteins and 6% glucose non treated (lane 1), incubated for 24 h (lane 2) and 48 h (lane 3). 0,5% hazelnut proteins with 0,5% wheat proteins and 6% glucose non treated (lane 4), incubated for 24 h (lane 5) and for 48 h (lane 6). 0,25% hazelnut proteins with 0,75% wheat proteins and 6% glucose non treated (lane 7), incubated for 24 h (lane 8) and 48 h (lane 9). Molecular weight marker (lane 10)

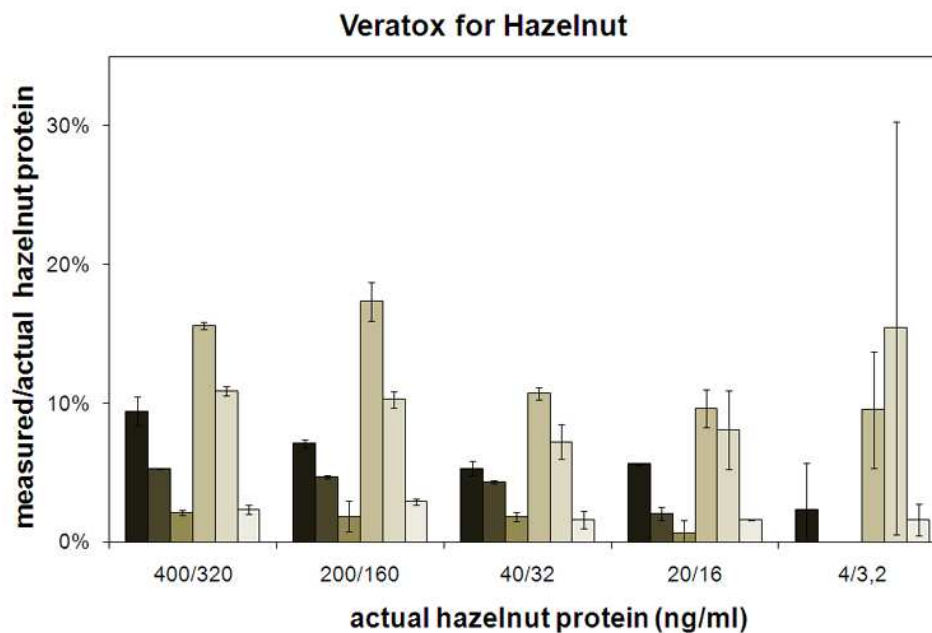


Figure 4 Ratio of measured over actual hazelnut protein concentration (Y-axis) in different ELISA kits after in duplo analysis of a dilution series (X-axis) of a reference hazelnut protein sample (darkest) and after heating in the presence or absence of glucose and in the absence (left concentration 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 sample, - reference heated for 48 h, - reference heated for 48 h in the presence of glucose, - 0.25% hazelnut proteins and 0.75% soluble wheat proteins, - 0.25% hazelnut proteins and 0.75% soluble wheat proteins heated for 48 h, - 0.25% hazelnut proteins and 0.75% soluble wheat proteins heated for 48 h in the presence of glucose

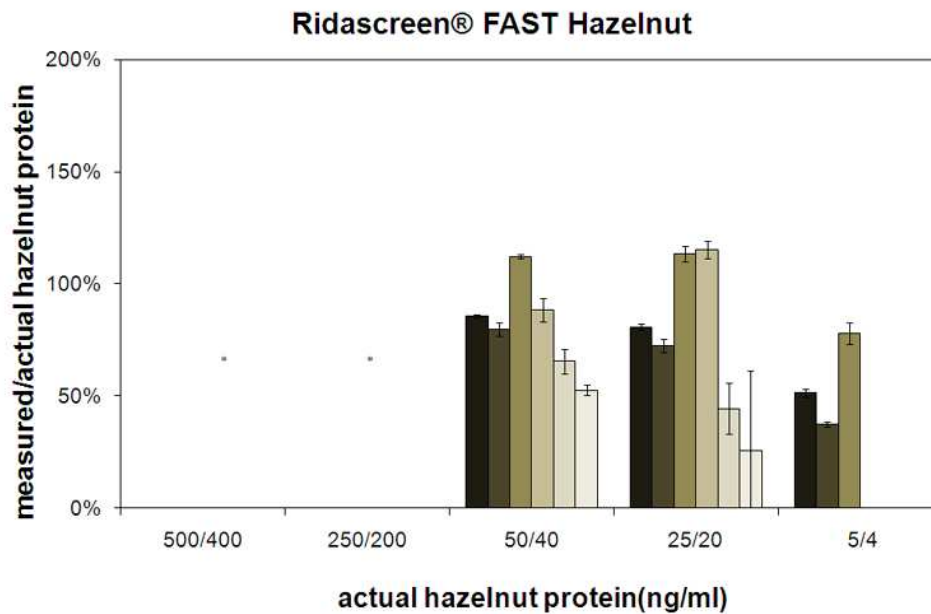


Figure 4 Ratio of measured over actual hazelnut protein concentration (Y-axis) in different ELISA kits after in duplo analysis of a dilution series (X-axis) of a reference hazelnut protein sample (darkest) and after heating in the presence or absence of glucose and in the absence (left concentration 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 sample, - reference heated for 48 h, - reference heated for 48 h in the presence of glucose, - 0.25% hazelnut proteins and 0.75% soluble wheat proteins, - 0.25% hazelnut proteins and 0.75% soluble wheat proteins heated for 48 h, - 0.25% hazelnut proteins and 0.75% soluble wheat proteins heated for 48 h in the presence of glucose

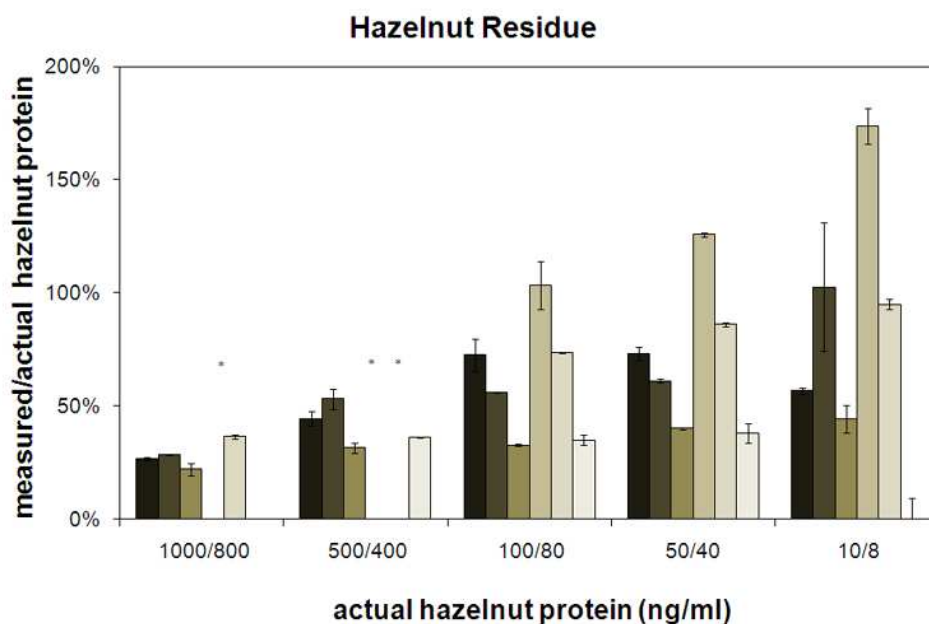


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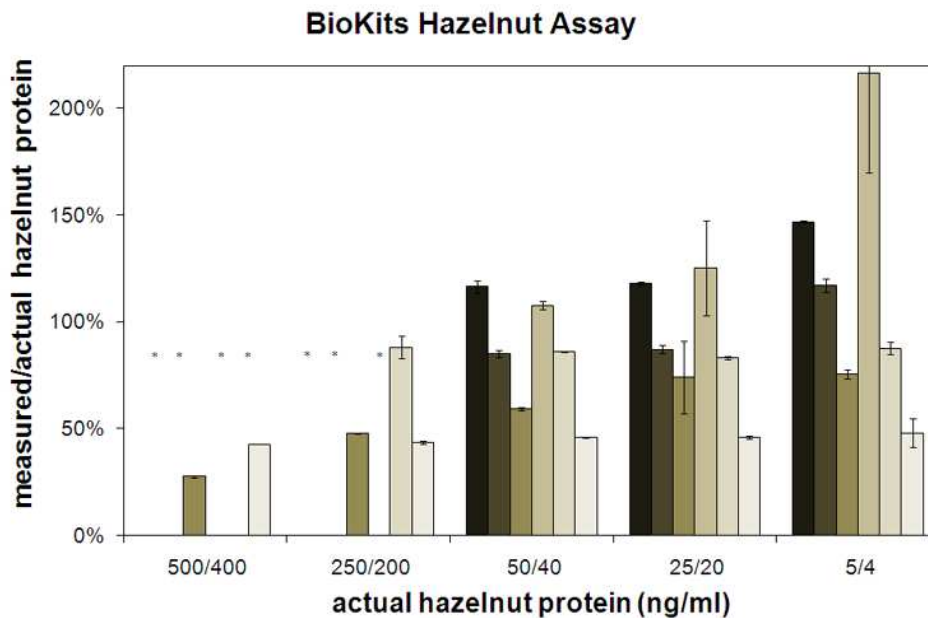


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