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Effect of protein glycation in the presence or absence of wheat proteins on detection of soybean proteins by commercial ELISA

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Abstract

2 Soybean (*Glycine max*) is the world's primary provider of protein and oil and is widely
3 used in foodstuffs. However, the use of soybean in foodstuffs might pose a serious threat to
4 the allergic consumers since some proteins can cause allergic reactions. To date, mostly
5 ELISA methods are used for testing contamination of foodstuffs with soybean. In view of the
6 complexity regarding allergen detection in foodstuffs and appropriate food product labelling,
7 the aim of the present study was to investigate the impact of the Maillard reaction on the
8 detectability of soybean proteins using commercial ELISA kits. Accumulation of protein
9 bound carbonyls, modification of reactive lysine residues and severe aggregation as a result of
10 incubation with glucose, in the presence or absence of soluble wheat proteins, were recorded.
11 Moreover, detection of soybean proteins by means of three commercial ELISA kits was
12 strongly altered and was highly dependent on the type of kit used.

14 **Keywords:** Soybean allergy, Maillard reaction, ELISA, protein bound carbonyls, protein
15 aggregation, reactive lysine.

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Introduction

18 Food allergy is an abnormal immunological response due to sensitization to a food
19 component. With its increasing incidence over the last years, it represents an important health
20 problem, especially in industrialized countries, where it has been estimated to affect about 2%
21 of the adult population and up to 8% of children (Monaci et al. 2006). Together with hen's
22 egg, cow's milk, fish, crustacea, peanut, tree nuts and wheat, soybeans belong to the 'big
23 eight', being responsible for 90% of all allergic reactions (Report of the FAO 1995). It also
24 belongs to the list of 14 allergenic products which have to be declared on the label of a food
25 product when present as an ingredient according to Directive 2007/68/EU (European
26 Parliament and Council Directive 2007/68/EC 2007).

30 Soybeans (*Glycine max*) contain about 35% of proteins and at least 21 proteins have
31 been shown to be IgE-reactive. Soybean allergy can occur as a result of primary sensitization,
32 but can also be related to birch pollen allergy through structural similarities between the birch
33 pollen allergen *Bet v 1* and the corresponding protein *Gly m 4* in soybeans (secondary allergy)
34 (Ballmer-Weber and Vieths 2008; Mittag et al. 2004). The symptoms associated with soybean
35 allergy are mostly mild, such as the Oral Allergy Syndrome (OAS), however, severe reactions
36 have also been described (Foucard and Yman 1999; Kleine-Tebbe et al. 2002; Mittag et al.
37 2004). The incidence of soybean allergy has increased during the last years probably because
38 more products are being produced with soybean (Wilson et al. 2005). Soybean is used as an
39 ingredient in a broad range of food products, both for its texturizing and nutritional benefits.
40 Due to its diverse functional properties, such as water and fat absorption, emulsification,
41 foaming and gelation, soybean proteins are often included during food processing steps.
42 However, extensive use of soybean proteins in foods might pose a serious threat to the
43 allergic consumers due to unintentional contamination which might take place during food

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3 processing. This can affect the safety of food products and lead to the presence of hidden
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6 42 allergens, therefore making avoidance diets increasingly challenging.
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8 Various approaches have been applied to reduce the allergenicity of soybean proteins.
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10 44 Enzymatic hydrolysis through fermentation is often applied to produce hypo-allergenic
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12 formulas, but is strongly dependent on the extent of the hydrolysis degree (Frias et al. 2008;
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15 46 Tsuji et al. 1997; Yamanishi et al. 1995). Reduction of soybean allergenicity has also been
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17 achieved by allergen conjugation with polysaccharides (Arita et al. 2001; Babiker et al. 1998).
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19 48 Similarly, the impact of protein modifications occurring as a result of the multitude of
20
21 chemical reactions occurring during food processing has been studied as well (Burks et al.
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24 50 1991; Codina et al. 1998; Muller et al. 2000). In contrast to these studies focusing on the
25
26 impact of soy protein modification on the antigenicity, only limited studies are available with
27
28 respect to the impact of these changes. In addition, the impact of these changes on the
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30 52 detectability using immunochemically methods, which are typically used by the industry and
31
32 enforcement agencies (Bando et al. 1998; Tsuji et al. 1995) has rarely been studied in a
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34 54 systematic manner for soybean protein.. The above-mentioned methods are based on antibody
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36 – antigen interactions Therefore, immunoassays are subjected to complications due to the
37
38 56 matrix effects, which may result in erroneous results. Moreover, the interaction of food
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40 allergens with other food components, which are expected to occur during thermal processing,
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42 58 may lead to changes in the allergen structure and might hinder their detection by the
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44 immunological methods. Therefore, it is important to gain knowledge on the influence of food
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46 60 processing on the tertiary structure of food allergens and the changes occurring at the amino
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48 acid level as a result of chemical reactions taking place during processing. Moreover,
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50 information on the further impact of these changes on the allergenic potential and the
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52 62 detectability of the allergens is invaluable. Van de Lagemaat *et al.* previously reported
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54 reductions of the antigenicity of soybean proteins after *in vitro* glycation using an in-house
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3 66 developed ELISA assay (de Lagemaat et al. 2007). In this study the impact of glycation in the
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5 presence or absence of other proteins on the detectability of soybean proteins by commercial
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8 68 kits was investigated. A similar study on hazelnut proteins already showed that the detection
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10 was drastically affected as a result of protein glycation (manuscript submitted).
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Materials and methods

72 *Reagents and standards*

Soluble wheat proteins “Meripro 711” were kindly provided by Syral Belgium N.V. (Aalst, Belgium). Chemicals and standards of analytical grade were obtained from Sigma-Aldrich (Bornem, Belgium), VWR (Leuven, Belgium) and Acros Organics (Geel, Belgium). Solvents were of analytical grade from Chem-Lab (Zedelgem, Belgium), except for hexane which was of technical grade. Gel and standards for SDS-PAGE were from Bio-Rad (Nazareth, Belgium) while the Krypton protein stain was provided by Thermo Scientific (Aalst, Belgium). The commercial ELISA kits used were Veratox® Soy Allergen (Neogen, Lansing, USA), BioKits Soya Allergen Assay (Tepnel, Flintshire, UK) and Soy Residue (ELISA Systems, Queensland, Australia).

82 *Soybean protein extraction*

Soybeans were provided by Alpro (Wevelgem, Belgium) and Cargill (Mechelen, Belgium). A mixture was made by taking equal amounts of each kind. Soybeans were frozen with liquid nitrogen and ground in a two-step process, first with a blender (Moulinex, France) and secondly with an Ultra Turrax t25 (IKA, Wilmington, US).

Soybean protein extracts were obtained according to the protocol described by Yeung et al. with slight modifications (Yeung and Collins 1996). Briefly, 100 g of ground beans were defatted with four times 1 L technical hexane. After an overnight evaporation of the solvent at ambient temperature, 10 g defatted powder was mixed with 100 ml of extraction buffer and mixed at 4°C over night. Protein extraction buffer consisted of a 1 M NaCl, 1.47 mM KH_2PO_4 , 2.7 mM KCl and 10 mM Na_2HPO_4 . Sodium azide 0.2 g L^{-1} was added to the extraction buffer to avoid microbial growth. Further a mixture of protease inhibitors (2 mM

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3 94 ethylenediaminetetraacetic acid (EDTA), 5 mM sodium diethyldithiocarbamate (DIECA), 0.5
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5 mM benzamidine hydrochloride (BAHC) and 0.2 mM phenylmethylsulfonyl fluoride
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7 (PMSF)) was added to the extraction buffer in order to increase the stability of the protein
8 96 isolates during extraction and storage (Bjorksten et al. 1980). The protein mixtures were
9
10 further centrifuged for 1 h at 4°C and 20000 g to separate the lees. Subsequently, the clear
11
12 supernatants were dialyzed (Spectra/Por 3 membrane tubing, MWCO 3.5 kDa) using the same
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14 extraction buffer containing only 0.137 M NaCl, for 48 h at 4°C and then filtered through a
15
16 100 0.45 µm membrane filter (Puradisc FP 30, Novolab, Belgium). Finally, the protein solutions
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19 were lyophilized and stored at -20°C until further use. This extract will further on in this
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21 paper be considered as the reference extract.
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28 104 ***Maillard reaction in the absence of soluble wheat proteins***

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30 Before treatment, soybean protein extracts were dialyzed against the 0.1M phosphate
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32 106 buffer pH 7.4 to remove the nitrogen containing additives (Spectra/Por 3 membrane tubing,
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34 MWCO 3.5 kDa). The reaction systems were prepared in the same phosphate buffer pH 7.4
35
36 by making a solution containing 1% (w/v) soybean proteins and 6% (w/v) glucose. The
37
38 108 reaction systems in closed falcon tubes were incubated in a water bath at 70°C for up to 48 h.
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41 A reaction system without glucose was used as control. At different time periods, samples
42
43 110 were retrieved from the water bath and placed in an ice bath to stop the reaction. Further, the
44
45 modified protein solutions were immediately used for the determination of reactive lysine
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47 112 residues, protein bound carbonyls and free amino groups. Aliquots for SDS-PAGE were
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49 frozen at -20°C until further use.
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55 116 ***Maillard reaction in the presence of soluble wheat proteins***

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58 116 Two types of model systems were considered. One model system contained 0.25%
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60 (w/v) of soybean proteins with 0.75% (w/v) of wheat proteins and 6% (w/v) of glucose in the

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3 118 same 0.1 M phosphate buffer pH 7.4. Another model system contained 0.5% (w/v) soybean
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5 proteins with 0.5% (w/v) wheat proteins and 6% (w/v) glucose. Respective model systems
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7
8 120 without glucose were prepared as control. Further these systems were treated similarly as the
9
10 model systems containing no soluble wheat proteins.
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12 122 ***Assessment of the Maillard reaction***

13 14 15 16 17 *Determination of protein bound carbonyls*

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20 124 Protein bound carbonyls were measured after converting them to the corresponding 2,
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22 4- dinitrophenylhydrazones. Briefly, 300 μ l protein solution was incubated with 400 μ l 2,4-
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24 126 dinitrophenylhydrazine (DNPH) 10 mM in 2 M HCl. Further, the proteins were precipitated
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26 with 10% trichloroacetic acid (final concentration) and thoroughly washed with 1 ml
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28 128 ethanol/ethyl acetate (1:1) after derivatization; hence any unreacted DNPH or DNPH bound to
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30 any other components than protein was removed. Finally the pellet was redissolved in 8 M
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32 130 urea and absorbance was measured at 370 nm using a Bio-Rad Benchmark Plus microplate
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34 spectrophotometer (Nazareth, Belgium). The carbonyl content was calculated using a molar
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36 132 absorption coefficient of 22000 M⁻¹ cm⁻¹ (Levine et al. 1994).
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42 *Loss of reactive lysine residues*

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45 134 The loss of reactive lysine residues was monitored using derivatization with *ortho*-
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47 phthaldialdehyde (OPA) in the presence of 2-mercaptoethanol which yields a fluorescent
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49 136 product with a maximum excitation wavelength of 340 nm and emission of 450 nm using a
50
51 Spectramax Gemini XPS fluorimeter from Molecular Devices (Brussels, Belgium) (Ferrer et
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53 al. 2003). A standard of β -casein was used to prepare a calibration curve and samples were
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55 138 pre-treated and analyzed using a fluorimeter as previously described (Morales et al. 1996).
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3 140 *SDS-polyacrylamide gel electrophoresis (SDS-PAGE)*
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6 Protein cross-linking or fragmentation was analyzed by SDS-PAGE under reducing
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8 142 conditions. A 4% acrylamide stacking gel and a 10% acrylamide resolving gel were used for
9 protein separation. An aliquot of 10 μ L sample containing 6 μ g of protein was added to 10 μ l
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11 Laemmli sample buffer containing β -mercaptoethanol and heated at 95°C for 5 min. The
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13 144 samples were further loaded on the gel along with the molecular weight marker. Precision
14 plus protein unstained standard was used as a reference to determine the molecular weight of
15
16 146 proteins of interest. Krypton fluorescence staining was used for bands visualization. The gels
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18 148 were further read using the Molecular Imager PharosFX Plus from Bio Rad (Nazareth,
19 Belgium) where the molecular weights of the proteins were recorded.
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28 150 *Protein determination*
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31 Protein determination was performed by determining the nitrogen content according to
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33 152 the Kjeldahl procedure (AOAC Official Method 981.10 1981). A multiplication factor of 5.71
34 was used to convert nitrogen content to soybean protein content, and a factor of 6.25 to
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36 154 convert to soybean protein content for the mixtures of soybean and wheat protein. The non
37 protein nitrogen (NPN) fraction was determined in the supernatant after a previous protein
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39 156 precipitation with 15% TCA (final concentration).
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46 *ELISA assays*
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49 158 Samples of the above-described model systems were analyzed using different
50 commercial ELISA kits: Veratox® Soy Allergen, BioKits Soya Allergen and Soy Residue.
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52 160 These samples include the glucose-free model system containing only soybean proteins
53 (system 1), the model system containing soybean proteins with glucose (system 2), the two
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55 162 model systems containing different ratio's of soybean proteins and soluble wheat proteins
56 (0,5% soybean proteins + 0,5% soluble wheat proteins (system 3) and 0,25% soybean proteins
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3 164 + 0,75% soluble wheat proteins (system 4), respectively) and the two model systems 3 and 4
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5 containing different ratio's of soybean proteins and soluble wheat proteins, in the addition of
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8 166 6% glucose ((system 5) resp. (system 6)). Of the different model systems, samples retrieved
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10 after 0, 24 and 48 h incubation at 70°C were analyzed. The untreated sample (= 0 hours of
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12 168 thermal treatment) of the glucose-free model system (1) containing only soybean proteins is
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14 referred to as the reference sample. For each sample of the different model systems a dilution
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17 170 series was made (0 – 50 – 100 – 500 – 1500 – 1500 ng/ml soybean protein) in the extraction
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19 buffer/dilution solution of the respective kit. For the BioKits Soya Allergen Assay additional
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22 172 dilutions had to be analyzed. The actual concentrations are the concentrations of soybean
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24 proteins based on the absolute protein determination using the Kjeldahl procedure and do not
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27 174 include the wheat proteins that were added in some of the model systems. The dilution series
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29 were analyzed *in duplo* in a similar manner as sample extracts according to the respective
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32 176 manufacturers' manuals. Absorbance measurements were made in a microtiter plate reader
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34 (Multiskan MCC/340, Titertek, Huntsville, Alabama). Each kit was calibrated using the
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36 178 respective standard protein solutions included in each kit. The absorbance values were fitted
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38 to the protein concentration of the standard solutions, according to a four-parameter-logistic
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41 180 dose-response curve corresponding to following equation (2.1). This equation presents a
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43 standard way for the expression of immunoassay data, where a is the maximum dose (protein
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45 concentration), b is the minimum concentration, c is the E₅₀-value (concentration at half-
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47 182 maximal saturation), d is the slope of the curve and $conc$ is the concentration of the standard
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51 184 or sample.

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

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3 186 Only the data obtained within the range of the respective standard curve were
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5 considered in the results. In case of the Veratox® Soy Allergen kit, the kit's procedure
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8 188 prescribes to fit the data to log-logit function, which is a linearization of the four-parameter
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10 logistic function. For the Soy Residue and the BioKits Soya Allergen Assay no suggestion is
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12 190 made as to which function to use to fit the data. Data above or below the calibration range are
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14 indicated by an asterisk (*) or a 'zero' (0), respectively, in the charts.
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192 **Results**

194 Progress of the Maillard reaction

194 An increase in protein bound carbonyl amounts as result of the incubation in the
195 presence of glucose has been recorded. A 5 fold increase in protein bound carbonyls was
196 recorded after 24 h incubation of 1% soybean with glucose while after 48 h incubation a 10
197 fold increase was observed (**Figure 1**). For the model system containing no glucose a 2 time
198 increase was observed. Similarly, an increase in protein bound carbonyls was observed when
199 soluble wheat proteins were added to the model systems. After 48 h incubation with glucose a
200 7 fold increase in protein bound carbonyls was observed in the case of the model system
201 containing 0.5% soybean proteins. For the model system containing 0.25% soybean proteins a
202 4 fold increase was observed.

203 Further, changes in the reactive lysine residues have been recorded. About 80%
204 decrease of reactive lysine residues has been recorded upon incubation of 1% soybean
205 proteins with glucose (**Figure 2**). Interestingly, a 23% decrease in reactive lysine residues
206 after 6 h incubation in the absence of glucose was also recorded. A further decrease in
207 reactive lysine residues was however not recorded, while in the presence of glucose, a steady
208 decrease was obtained throughout the whole incubation experiment. Further, about 90% of the
209 reactive lysine residues were modified in the model system containing 0.5% soybean proteins
210 after 48 h of incubation with glucose while for the model system containing 0.25% soybean
211 proteins almost all the reactive lysine residues were modified.

212 **Figure 3** shows the SDS-PAGE pattern of the native and glycated soybean proteins.
213 Changes in the SDS-PAGE pattern of the soybean proteins incubated in the absence of
214 glucose were observed (lane 1-3). The intensity of the bands representing the α -, α - and β -
215 chain of the Gly m 5 allergen with the molecular weight of 70, 74 and 50kDa respectively,
216 decreased markedly with the incubation time (I.U.I.S.Allergen Nomenclature 2009;

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3 UniProtKB/Swiss-Prot 2010). However, the acid and basic subunits of the *Gly m 6* allergen
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5 218 (32 and 19 kDa, respectively) remained stable upon the whole incubation time. More severe
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7 changes were observed however, in the SDS-PAGE pattern of the soybean proteins incubated
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10 220 with glucose either in the presence or absence of soluble wheat proteins. Most of the
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12 allergenic proteins have been affected as manifested by the loss of intensity of their respective
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14
15 222 bands. Similarly as with the incubation in the absence of glucose, the α -, α' - and β -chain of
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17 the *Gly m 5* allergen were affected the most with the acid and basic subunits of the *Gly m 6*
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19 224 allergen being the most resistant. The respective bands of the *Gly m 6* allergen were still
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21 visible after 24 h incubation in the presence of glucose although with slightly faded
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23
24 226 intensity. All the other proteins were highly modified as evidenced by the smearing towards
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26 the top of the gel which progressed with the incubation time. After 48 h of incubation with
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29 228 glucose only the bands corresponding to the acid and basic subunits of the *Gly m 6* allergen
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31 were still slightly visible. Aggregates with masses of over 300 kDa were also formed which
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34 230 hindered their migration into the resolving gel.

37 ***Detection with commercial ELISA kits***

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40 232 The soybean protein concentration of the standards provided by each individual kit is
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42 expressed in parts per million (ppm) which equals $\text{ng analyte mg}^{-1}$ of food product. When
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44 234 testing the commercial kits, the objective was to compare the actual soybean protein
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46 concentration of the analyzed samples with the measured concentration, derived from the
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49 236 calibration curve obtained with the kit's standards. To this end, the concentration of the kits
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51 standards was converted to the same dimension of that from the model system samples. This
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54 238 was done according to formula 3.1, considering the weight of the sample (mg) extracted by a
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56 particular volume of extraction buffer (mL).

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$$\frac{\text{ng analyte}}{\text{mg food}} (\text{ppm}) \times \frac{\text{weight of extracted sample (mg)}}{\text{volume of extraction buffer used (ml)}} = \frac{\text{ng analyte}}{\text{ml solution}} \quad (3.1)$$

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For the evaluation of the kits performance, dilution series of the soy protein solutions obtained from the six different model systems were applied in the appropriate concentration range of each kit. The obtained absorbance values were converted to a measured soy protein concentration, which was compared with the actual protein concentration presents in each diluted solution (Figure 4).

248 *Biokits Soya Allergen*

With the standard solutions included in the BioKits Soya Allergen assay, a calibration curve was constructed according to the four-parameter logistic model, ranging from zero to 200 ng mL⁻¹ soybean protein. The soybean protein concentration in the reference samples was overestimated on average by 290% in a concentration dependent manner (**Figure 4A**). Therefore, at the highest concentrations absorbance values exceeding those obtained in the calibration curve were observed. Upon heating at 70°C without glucose for 24 h the signal decreased with 14% (not shown) and an additional 8% when the treatment was extended up to 48 h. When soluble wheat proteins were added to the model system the average overestimation of the concentration of the samples was diminished to 250%, irrespective the soy/wheat protein ratio. However, at the lowest soybean/wheat protein ratio, a sharper decline in the measured soybean protein concentration was observed upon heating; after 48 h the signal dropped on average 38% (0.75% soluble wheat proteins) compared to an average of 27% (0.5% soluble wheat proteins, not shown). Addition of glucose resulted in a further decrease of the measured concentration compared to the samples treated without glucose; the signal was declined with 60% after 48 h heat-treatment.

264 *Veratox® Soy Allergen*

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6 A calibration curve was established with the standard solutions provided in the
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9 266 Veratox® Soy Allergen kit. The standard consists of Soy Protein Isolate and covers a range
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11 from zero to 4000 ng mL⁻¹ after conversion of the concentration expressed in ng g⁻¹,
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13 268 according to formula 3.1. As can be observed from **Figure 4B**, the actual soybean protein
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15 concentration present in the reference solution and its dilutions was highly overestimated on
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17 average by 300%. Therefore, the obtained absorbance values for the reference samples with
18 270 1500 and 1000 ng mL⁻¹ soybean protein were beyond the range of the calibration curve and
19
20 were not included in Figure 4B. Upon the presence of soluble wheat proteins in the model
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22 272 system the overestimation was even increased up to on average 400% (0.5% soybean protein
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24 + 0.5% soluble wheat proteins, not shown) or on average 600% (0.25% soybean proteins +
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26 274 + 0.75% soluble wheat proteins). However, already after heating the reference sample for 24 h
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28 in the absence of glucose the signal disappeared completely. In the presence of soluble wheat
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30 276 proteins only 1% (0.5% soluble wheat proteins) or 8% (0.75% soluble wheat proteins) of the
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32 soybean proteins was detected after 24 h heating (not shown) and extension to 48 h lead to a
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34 complete loss of signal. Heating the proteins in the presence of glucose confirmed the lack of
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36 278 robustness of the kits.
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44 *Soy Residue*

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47 282 In the Soy Residue assay the soybean trypsin inhibitor is used as the analytical target.
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49 The kit includes standard solutions containing 5, 2.5, 1 and 0 ppm soybean protein
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51 284 corresponding to 500, 250, 100 and 0 ng mL⁻¹ soybean protein after conversion which were
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53 used to construct a four-parameter logistic calibration curve. The 5 and 2.5 ppm standards
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55 286 returned equal absorbance values, amounting 2.8-3, consequently quantification can only be
56
57 performed up to a level of 2.5 ppm. In addition, the 1 ppm standard, being the lowest standard
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59 288 containing the analyte, returned a quite high absorbance value of 2.5. Therefore, it was
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1
2
3 decided to dilute the available standards provided with the kit in order to obtain a more
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5
6 290 suitable calibration curve. The protein concentration of the native reference sample was
7
8 underestimated for the samples containing 50 and 100 ng mL⁻¹ soybean protein in a
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10 292 concentration depend manner with on average 12% (**Figure 4C**). Exceeding the calibration
11
12 range, the reference samples containing 500, 1000 and 1500 ng mL⁻¹ soybean protein could
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14
15 294 not be considered and the latter two were not included in Figure 4C. Upon heating without
16
17 glucose for 24 h at 70°C, only about 2% of the proteins could be detected (not shown) with an
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19 296 additional decrease to only 1% when extended to 48 h. When soluble wheat proteins were
20
21 added to the system, the measured concentration was lower compared to the reference sample
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23
24 298 (15 – 20%); in contrast to the observations described for the Veratox® Soy Allergen, a lower
25
26 signal was measured when more wheat proteins were added. Also here an almost complete
27
28
29 300 loss of signal was observed after heating in the presence of soluble wheat proteins, with only
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31 1% of the soybean proteins being detected after 24 h (not shown) and 0.2% after 48 h.
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34 302 Similarly, no additional effect of adding glucose during the heat-treatment could be seen.
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Discussion

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7 304 In this study, different commercial ELISA kits for the detection of soy proteins and the
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9 influence of protein glycation hereon was investigated. Application of the reference soybean
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11 306 protein solution in the various kits resulted in an extreme overestimation of the actual protein
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13 concentration in the Veratox Soy Allergen and BioKits Soya assays, while for the Soy
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15
16 308 Residue assay an underestimation was recorded. In addition, the amount of soybean proteins
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18 that was measured was dependent on the concentration in the sample that was applied. Similar
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21 310 observations have been made in a validation study of five commercial ELISA kits for peanut
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23 detection (Poms et al. 2005). A possible explanation for the differences between the actual
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26 312 and the measured soybean protein concentration that were obtained with the different kits
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28 could be found in the nature of the standard solutions of the respective kits. In the Soy
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30
31 314 Residue kit a single protein, namely the Soy Trypsin Inhibitor, is used as the target analyte.
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33 Possibly the factor used to convert the soy trypsin inhibitor concentration to total soy protein
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35 316 concentration is not the same as the actual proportion soy trypsin inhibitor present in the
36
37 soybean protein extract used in this study. Strategically, the choice for a single marker protein
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40 318 for the detection of soybean proteins in processed food products is especially risky in terms of
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42 susceptibility to protein modification and its impact on the detection, as was moreover
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45 320 demonstrated in this study. The high overestimation of the actual soy protein with the BioKits
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47 Soya Allergen assay demonstrate that the antibodies that are used to develop the assay
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49
50 322 recognize better the proteins that are present in the soybean protein extract used in this study
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52 than the ones present in the standard solutions of the kit. This could be due to the use of
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54 324 different soybean varieties to produce the protein extract. Immunochemical activity of peanut
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56 proteins has also been shown to depend on the peanut variety that is used (De Meulenaer et al.
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58
59 326 2005). These observations indicate that the test results heavily depend on the assay that is
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3 used and urge the need for reference materials for the development and validation of
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6 328 standardized methods.

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8 Upon heating the reference soybean protein solution in absence of glucose, a decreased
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10 330 detection was observed in the BioKits Soya Allergen, however, still overestimating the actual
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12 soybean protein concentration. In contrast, both the Veratox Soy Allergen and the Soy
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15 332 Residue assay lacked total robustness. The complete loss of detectability can probably be due
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17 to the denaturation of soybean proteins especially because only minor changes in terms of
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20 334 formation of protein carbonyls and losses of lysine residues were observed as compared to the
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22 changes observed in the model systems containing glucose (**Figure 1&2**). However, the
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24
25 336 observed loss of intensity of the *Gly m 5* allergen upon heating without glucose, indicating
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27 denaturation, might explain the lowered detection by the BioKits Soya Allergen Assay
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30 338 (*Kitabatake et al. 1989*). The *Gly m 6* allergen, with a denaturation temperature of about 90°C
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32 could then account for the remaining detection. The Soy Trypsin Inhibitor shows a thermal
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34 340 transition at 60-65°C which could explain the decreased detection with the Soy Residue assay
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36 after heating at 70°C (*Roychaudhuri et al. 2004*). Although the denaturation of this protein has
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39 342 been shown to be reversible, fast cooling of the model systems, by putting the samples on ice
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41 after heat-treatment, could have prevented a proper refolding.

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43 344 When soybean proteins are present in a food product, protein cross-linking can occur
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45 with other (bulk) proteins, which could have an additional impact on the detection. Therefore
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47
48 346 the impact of heating soybean proteins in the presence of soluble wheat proteins was
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50 investigated as well. In a previous study on hazelnut proteins it was seen that soluble wheat
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53 348 proteins can have an additional impact (manuscript submitted). However, no additional
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55 impact on the detection of soybean proteins was seen when soluble wheat proteins were added
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58 350 to the model systems.

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3 During heating of the reference soybean proteins with glucose either with or without
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6 352 soluble wheat proteins, changes on molecular level took place as evidenced by a number of
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8 parameters investigated (**Figure 1-3**). As a consequence, these changes on protein level led to
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10 354 further decreases in detectability especially for the BioKits Soya Allergen Assay.
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12 Nevertheless, in the same kit, additional protein cross-linking with other soluble wheat
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15 356 proteins via the produced protein bound carbonyls during glycation, did not further affect the
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17 detectability of the soybean proteins. As a complete lack of robustness of the Veratox Soy
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19 358 Allergen and the Soy Residue was observed upon heating soybean proteins alone, no
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21 additional impact of their heating with glucose either in the presence or absence of soluble
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24 360 wheat proteins could be seen.
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27 The results presented show that the detection of soybean proteins by means of
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29 362 commercial ELISA kits is strongly influenced by processing. This implies that certain assays
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31 could falsely declare a processed food product as “free of soybean proteins”, while soybean
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34 364 proteins might still be present. Consequently, this holds a risk for the allergic consumer. In the
35
36 same time some other kits, such as BioKits Soya Allergen and Veratox Soy Allergen, show
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39 366 strong overestimation especially for the native soybean proteins which presents risks for the
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41 food industry where the product withdrawal from the market is based on the results obtained
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44 368 with similar commercial kits. In conclusion, there is still a lack of sensitive analytical methods
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46 for the detection of traces of allergens in processed foods and thus controls cannot be carried
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49 370 out in a reliable way. Moreover, in this study, by inducing Maillard reaction in soybean
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51 proteins in a buffered system, abstraction of possible consequences of protein insolubility and
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53 372 eventual extractability was made. This implies that, within a real food product, it might be
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55 expected that estimations of the contamination levels would be even more severely affected. It
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58 374 should therefore be emphasized that the tested ELISA assays can be useful to give an
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60 indication of the presence of contaminants, rather than an exact quantitative value of the

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3 376 contamination level. But even if such assays are used in a mere qualitative context, utmost
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5 care should be given with respect to the interpretation of the results.
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14 382 us the Veratox Soy Allergen ELISA kits for this study.
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3 488 **Figure captions**
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6 **Figure 1** Formation of protein bound carbonyls in soybean protein in the presence or absence
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8 490 of soluble wheat proteins due to incubation with glucose. The data points represent mean
9 values \pm SD of three independent determinations. \square 1% soybean proteins with 6% glucose; \circ
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11 492 0.5% soybean with 0.5% soluble wheat proteins and 6% glucose; Δ 0.25% soybean and
12 0.75% soluble wheat proteins and 6% glucose; \diamond 1% soybean proteins
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18 494 **Figure 2** Loss of available lysine due to incubation of soybean proteins in the presence or in
19 the absence of soluble wheat proteins with glucose, data points represent mean values \pm SD of
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21 four independent determinations. \square 1% soybean proteins with 6% glucose; \circ 0.5% soybean
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23 496 with 0.5% soluble wheat proteins and 6% glucose; Δ 0.25% soybean and 0.75% soluble wheat
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27 498 proteins and 6% glucose; \diamond 1% soybean proteins
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30 **Figure 3** SDS-PAGE pattern of the soybean proteins incubated in the presence or absence of
31
32 wheat proteins with glucose Lanes 1-3: 1% soybean proteins non-treated, 24 h and 48h
33 500 incubated resp.; lanes 4-6: 1 % soybean proteins and 6 % glucose non-treated, 24 h and 48 h
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37 502 incubated resp.; lanes 7-9: 0.5 % soybean proteins with 0.5 % wheat proteins and 6 % glucose
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39 non-treated, 24 h and 48 h incubated resp.; lanes 10-12: 0.25 % soybean proteins with 0.75 %
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41 504 wheat proteins and 6 % glucose non-treated, 24 h and 48 h incubated resp.; MWM -
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45 molecular weight marker
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48 506 **Figure 4** Ratio of measured over actual soybean protein concentration in different ELISA kits
49 of a dilution series of a reference soybean protein solution and after heating in the presence or
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51 absence of glucose and soluble wheat proteins. (* = absorbance value of sample above the
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53 508 calibration range, 0 = absorbance value of sample below the calibration range)
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57 510 \blacksquare -reference sample, \blacksquare -reference heated for 48 h, \blacksquare -reference heated for 48 h in the presence
58 of glucose, \blacksquare -0.25% soybean proteins and 0.75% soluble wheat proteins, \blacksquare -0.25% soybean
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512 proteins and 0.75% soluble wheat proteins heated for 48 h, □ -0.2 % soybean proteins and
0.75% soluble wheat proteins heated for 48 h in the presence of glucose

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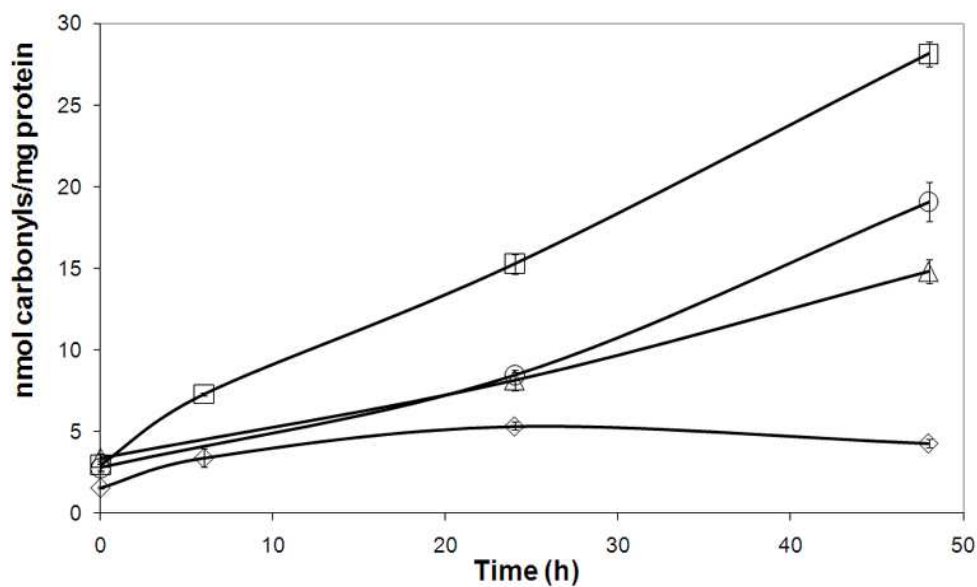


Figure 1 Formation of protein bound carbonyls in soybean protein in the presence or absence of soluble wheat proteins due to incubation with glucose. The data points represent mean values \pm SD of three independent determinations. \square 1% soybean proteins with 6% glucose; \circ 0.5% soybean with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

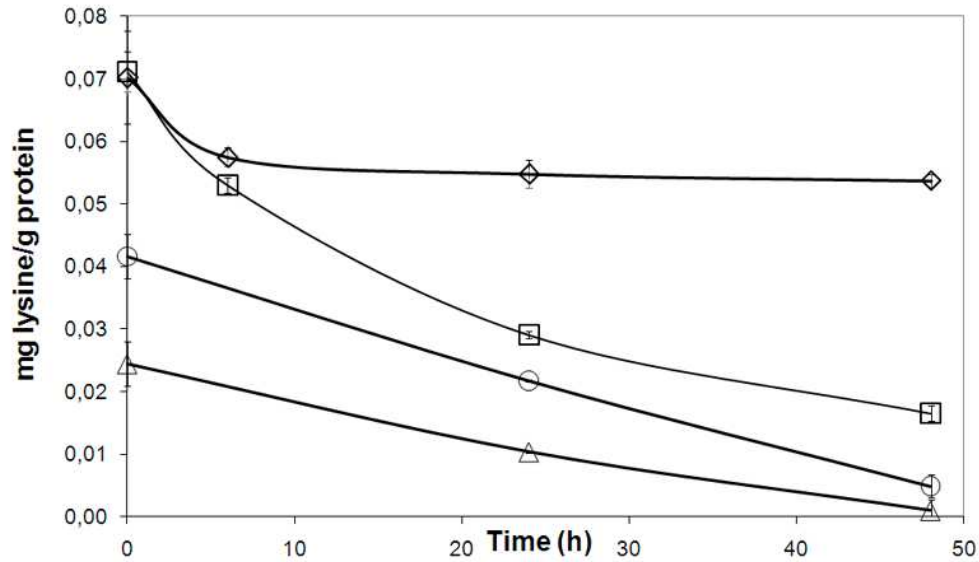


Figure 2 Loss of available lysine due to incubation of soybean proteins in the presence or in the absence of soluble wheat proteins with glucose, data points represent mean values \pm SD of four independent determinations. \square 1% soybean proteins with 6% glucose; \circ 0.5% soybean with 0.5% soluble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

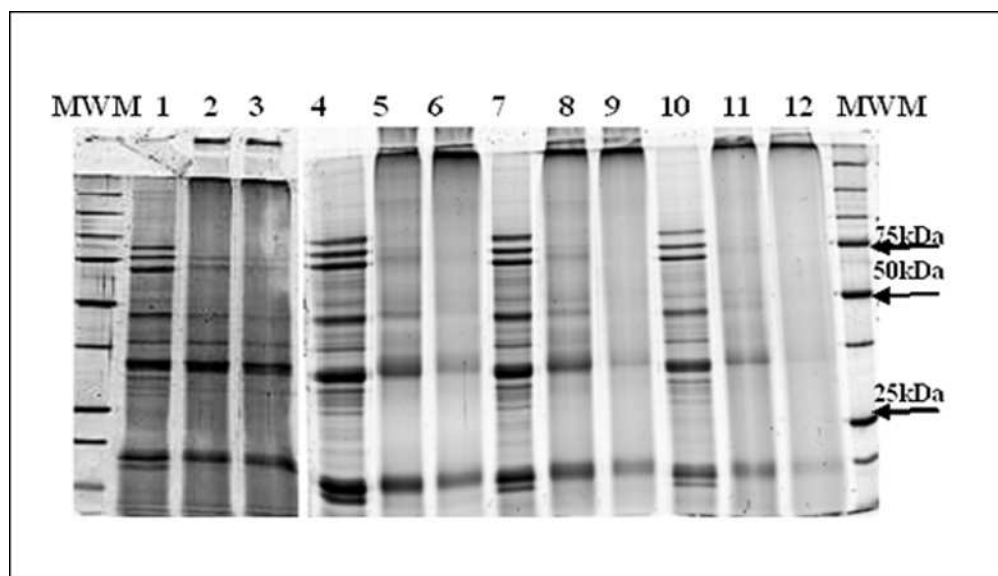


Figure 3 SDS-PAGE pattern of the soybean proteins incubated in the presence or absence of wheat proteins with glucose Lanes 1-3: 1% soybean proteins non-treated, 24 h and 48h incubated resp.; lanes 4-6: 1 % soybean proteins and 6 % glucose non-treated, 24 h and 48 h incubated resp.; lanes 7-9: 0.5 % soybean proteins with 0.5 % wheat proteins and 6 % glucose non-treated, 24 h and 48 h incubated resp.; lanes 10-12: 0.25 % soybean proteins with 0.75 % wheat proteins and 6 % glucose non-treated, 24 h and 48 h incubated resp.; MWM - molecular weight marker
99x57mm (199 x 199 DPI)

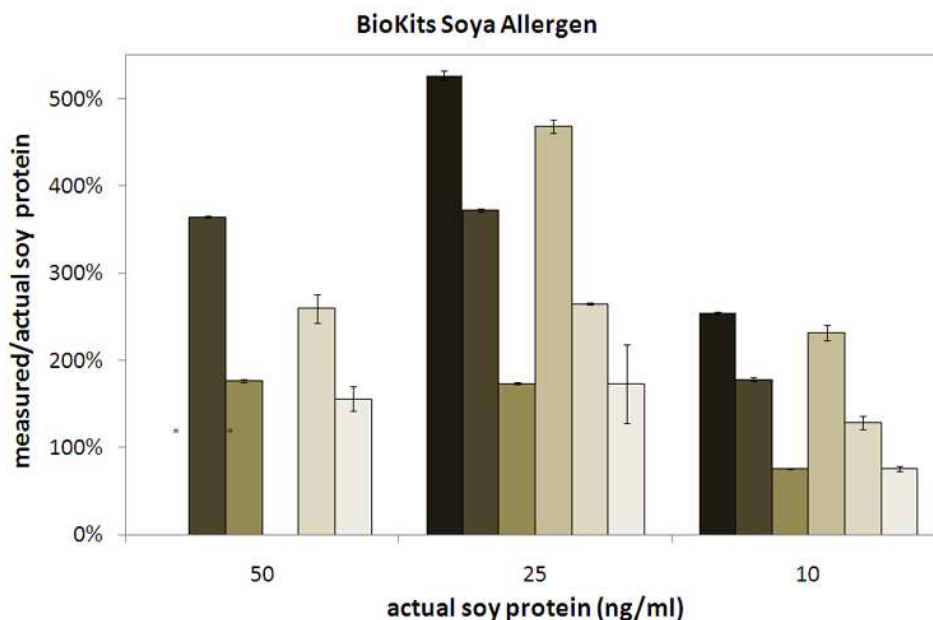


Figure 4 Ratio of measured over actual soybean protein concentration in different ELISA kits of a dilution series of a reference soybean protein solution and after heating in the presence or absence of glucose and soluble wheat proteins. (* = absorbance value of sample above the calibration range, 0 = absorbance value of sample below the calibration range)
 -reference sample, -reference heated for 48 h, -reference heated for 48 h in the presence of glucose, -0.25% soybean proteins and 0.75% soluble wheat proteins, -0.25% soybean proteins and 0.75% soluble wheat proteins heated for 48 h, -0.2 % soybean proteins and 0.75% soluble wheat proteins heated for 48 h in the presence of glucose

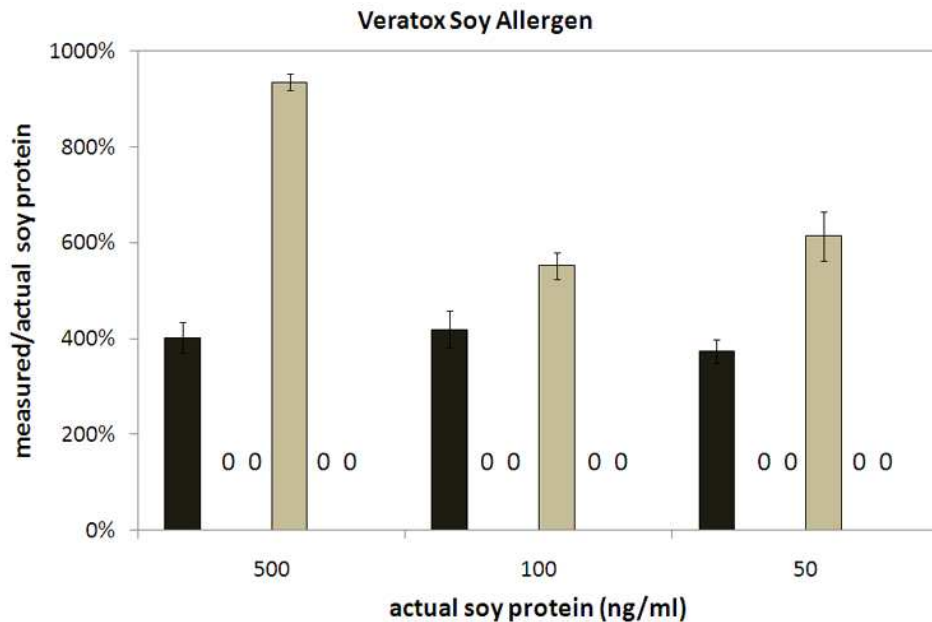


Figure 4 Ratio of measured over actual soybean protein concentration in different ELISA kits of a dilution series of a reference soybean protein solution and after heating in the presence or absence of glucose and soluble wheat proteins. (* = absorbance value of sample above the calibration range, 0 = absorbance value of sample below the calibration range)
 -reference sample, -reference heated for 48 h, -reference heated for 48 h in the presence of glucose, -0.25% soybean proteins and 0.75% soluble wheat proteins, -0.25% soybean proteins and 0.75% soluble wheat proteins heated for 48 h, -0.2 % soybean proteins and 0.75% soluble wheat proteins heated for 48 h in the presence of glucose

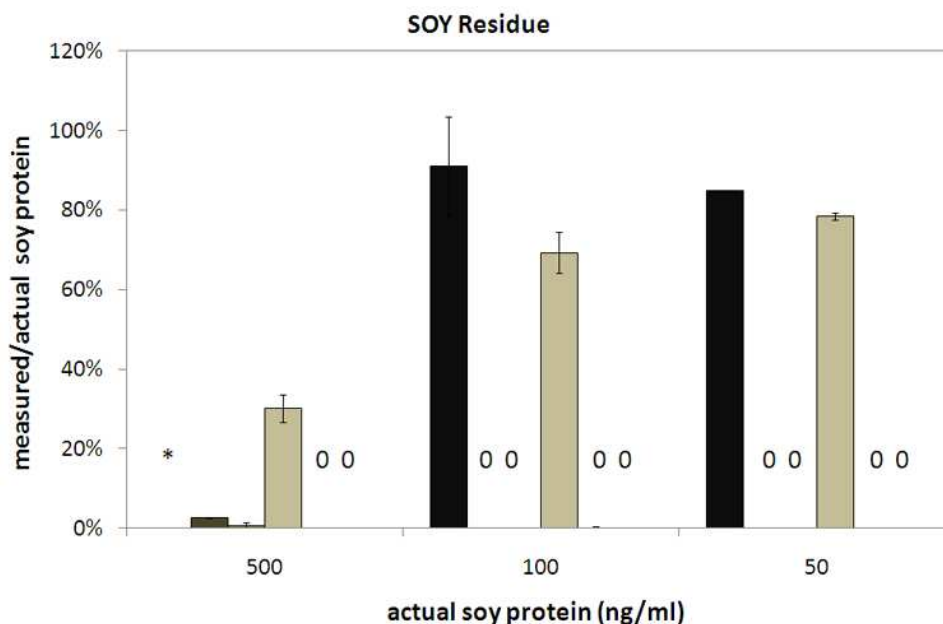


Figure 4 Ratio of measured over actual soybean protein concentration in different ELISA kits of a dilution series of a reference soybean protein solution and after heating in the presence or absence of glucose and soluble wheat proteins. (* = absorbance value of sample above the calibration range, 0 = absorbance value of sample below the calibration range)
 -reference sample, -reference heated for 48 h, -reference heated for 48 h in the presence of glucose, -0.25% soybean proteins and 0.75% soluble wheat proteins, -0.25% soybean proteins and 0.75% soluble wheat proteins heated for 48 h, -0.2 % soybean proteins and 0.75% soluble wheat proteins heated for 48 h in the presence of glucose