



Effects of processing on the recovery of food allergens from a model dark chocolate matrix



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ABSTRACT

To alleviate the risk to allergic consumers, it is crucial to improve factors affecting the detection of food allergens in processed chocolate products. This study evaluated processing effects on (1) recovery of peanut, egg, and milk allergens using five different extraction buffers, and (2) identification of specific allergenic proteins from extracts of incurred chocolate using allergen-specific antibodies and human allergic sera. Immunochemical staining with polyclonal antibodies showed that the addition of detergent or reducing agent improved extraction efficiency of peanut proteins, but not of egg and milk proteins. Tempering decreased antibody binding regardless of extractant. Detection of IgE-reactive peanut, egg, and milk allergens was differentially affected by tempering and extractant. Detection problems associated with matrix and processing effects may be overcome by the choice of extraction buffer and detecting antibody.

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1. Introduction

The recent growth in chocolate consumption is due, in part, to the nutritional benefits of its constituent components (Hii, Law, Suzannah, Misnawi, & Cloke, 2009; Rusconi & Conti, 2010). This has resulted in an increase in the number of chocolate-associated allergen safety recalls by the U.S. Food and Drug Administration. Chocolate components are not allergenic by themselves, although the amine in chocolate has been reported to cause symptoms such as migraine or hypertension (Settipane, 1987). However, allergenic material can be present in chocolate and chocolate products as a part of the product formulation or as an adulterant. For example, milk, sugar, nuts, or fruits are often added to chocolate to improve texture, flavour, taste or nutritional value. Among FDA food allergen recalls, milk-related recalls are the most common (Gendel & Zhu, 2013).

Allergic reactions are more common following the consumption of processed and mixed food products, as opposed to unprocessed and single ingredient foods (Grimshaw et al., 2003; Pele, Brohée, Anklaam, & Van Hengel, 2007), increasing the importance of accurate food labelling. Mislabeling and adulteration are the biggest threats to allergic consumers and strict avoidance cannot prevent the risk to allergic consumers of “hidden” allergens in complex foods. Cross contact, incomplete cleaning of production lines, or

improper package labelling are the major causes of inadvertent allergen exposures from chocolate products containing one or more allergens, as determined by surveillance studies (Pele et al., 2007; Spanjersberg, Knulst, Kruizinga, Van Duijn, & Houben, 2010).

Effective food allergen management plans rely on detection methods to develop appropriate labelling guidelines and assure consumer safety. The classic detection and quantification methods are antibody-based enzyme-linked immunosorbent assay (ELISA) and lateral flow, DNA-based polymerase chain reaction (PCR) and liquid chromatography–mass-spectrometric (LC–MS) (Kirsch et al., 2009). Quantitative LC–MS is more difficult to standardise and requires specialised equipment, making this method difficult for field application. Regardless of the detection method, effective sample preparation from complex processed food matrices is a challenging assay parameter.

Matrix components are prone to form covalent, ionic, and hydrogen bonds or hydrophobic interactions with target proteins. Dark chocolate is one of the more difficult food matrices because the components of chocolate, such as high fat, polyphenol, and tannin, can directly interfere with extraction and detection procedures. Food manufacturers utilise a variety of chocolate processing procedures including heating, chilling, melting, high pressure treatment, ultrafiltration, irradiation, hydrolysis and fermentation that lead to formation of variety of chemical rearrangements, including aldol condensation, polymerisation, cyclisation, and alkalisation, which enhance the flavour, colour, shelf life, bioavailability and nutritional

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value of chocolate (Afoakwa, 2010). Each of these processes can modify the structure and function of the chocolate components or generate interactions between chocolate components and other ingredients. Chocolate and chocolate products undergo a tempering procedure to improve texture, quality, and appearance. During tempering, the fat in chocolate influences the crystallisation of end products (Afoakwa, 2010), potentially masking allergen detection from a tempered chocolate matrix.

In addition to effects on chocolate matrix components, food processing can physically and chemically modify allergenic proteins themselves, or induce interactions between allergens and matrix components (Taylor, Nordlee, Niemann, & Lambrecht, 2009). Such modifications include unfolding, aggregation, fragmentation, hydrolysis, covalent modification, or total degradation, ultimately altering the protein epitopes that are responsible for eliciting allergic reactions or are the target of immunochemical detection methods. A well-defined heat-accelerated protein modification is the Maillard reaction between the carbonyl group of a reducing sugar in chocolate and the amino group of a protein (Davis, Smales, & James, 2001), resulting in the glycosylation of allergenic proteins. New allergen epitopes (neoepitopes) can also be revealed as result of the conformational changes in the allergenic protein by food processing. Therefore, processing can result in increased or decreased allergenicity of proteins either by the formation of new IgE binding epitopes or by the destruction of existing ones (Clare Mills, Sancho, Rigby, Jenkins, & Mackie, 2009; Nowak-Węgrzyn & Fiocchi, 2009). In addition to changes in allergenicity, processing procedures affect the extractability, solubility, and analysis of food allergens (Steinhoff, Fischer, & Paschke-Kratzin, 2011).

This study focused on the qualitative comparison of total protein and allergenic proteins extracted from a model food matrix. The selection of extraction solutions and conditions was based on conditions previously optimised for extraction of peanut, egg, and milk allergens (Cabanillas et al., 2012; Steinhoff et al., 2011; Watanabe et al., 2005; Westphal, Pereira, Raybourne, & Williams, 2004). Test extractants varied in pH, ionic strength, detergent/denaturant and reducing agent content. Total protein extraction efficiency and immunoreactivity of extracted allergenic proteins were determined using polyclonal antibodies raised against native and processed allergens and human allergic serum. The results of this study may have wider application for analysis of food allergens from other difficult food matrices.

2. Experimental

2.1. Food samples

Processed and unprocessed dark chocolate was studied. Control (0 ppm) and incurred chocolate (500 ppm) was prepared at FDA-CFSAN, National Center for Food Safety & Technology (Bedford Park, IL, USA) as described previously (Khuda et al., 2012). Chocolate was incurred with non-fat dry milk (NIST SRM#1549; National Institute of Standards and Technology, Gaithersburg, MD, USA), spray-dried whole egg powder (NIST SRM#8445) and light roasted peanut flour, 12% fat, product#521271 (Golden Peanut Company, Alpharetta, GA, USA). Scharffen Berger dark semisweet 62% Cacao chocolate (Robinson, IL, USA) was used because the manufacturer produces it on a dedicated line (peanut, egg and milk-free).

2.2. Chocolate extraction

Five different buffers were used for protein extraction of control and incurred chocolate samples: phosphate-buffered saline (PBS)-10 mM pH 7.4; high-salt buffer (HSB)-20 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 1 M NaCl pH 7.4; 0.1 M carbonate, pH 9.5; 0.05 M Tris-HCl, pH 8,

plus 0.5 M NaCl (Cabanillas et al., 2012); and HSB containing 1% sodium dodecyl sulphate (SDS) plus 7% 2-mercaptoethanol (ME). Triplicate 2 g portions of the 0 ppm, 500 ppm untempered and 500 ppm tempered samples were melted at 60 °C for 30 min, and extracted by vigorous mixing with 5 ml of buffer solution for 1 h at room temperature, then centrifuged at 20,000g for 30 min at 4 °C, and clarified by filtration through a 0.45 µm PVDF filter. Samples in HSB/SDS/2-ME were extracted overnight at room temperature. Extraction conditions used were previously optimised for egg, and milk, (Steinhoff et al., 2011; Watanabe et al., 2005; Westphal et al., 2004) and peanut (Cabanillas et al., 2012).

2.3. Protein quantification

Triplicate chocolate samples were extracted and triplicate protein levels per sample were determined using a Pierce 660 nm protein assay kit containing an ionic detergent compatibility reagent (IDCR) (Pierce Chemical Co, Rockford, IL, USA).

2.4. Preparation of allergens for antibody production and immunoblot

2.4.1. Peanut antigens

Ara h 1 from raw defatted peanut flour (Golden Peanut Company, product #521261) and Ara h 2, Ara h 3, and Ara h 6 from light roasted defatted peanut flour (Golden Peanut Company, product #521271) were purified at Protein Labs, Inc. (San Diego, CA, USA) using proprietary modifications of the methods of Koppelman et al. (2003) and Bernard et al. (2007). Ara h 1 was purified from raw peanut because of the difficulty in isolating sufficient quantities from roasted peanut, as has been demonstrated previously (Chruszcz et al., 2011). Raw peanut proteins, light roasted peanut proteins, and denatured peanut proteins were prepared as described previously (Yeung & Collins, 1996).

2.4.2. Egg antigens

Raw egg white was separated from yolk using cheese cloth and then extracted with PBS at in a shaking incubator (4 °C, 150 rpm) for 3 h. After centrifugation at 3000g for 10 min at 4 °C, the supernatant was dialyzed against PBS and filtered through 0.45 µm filters. Grade A large eggs (about 60 g each) were boiled in water for 10–15 min to prepare boiled egg white antigen. Boiled egg white was blended with PBS and extracted as above for raw egg white antigens. For preparing fried egg white antigens, egg white was pan fried at 200 °C surface temperature (as determined by a thermal couple) for 10 s. Fried egg white was suspended in PBS and extracted as above. Ovomucoid (Type III-O trypsin inhibitor), ovotransferrin and ovalbumin proteins from chicken egg white were purchased from Sigma-Aldrich (St. Louis, MO, USA) and further purified by high pressure liquid chromatography using a Waters Corporation (Columbia, MD, USA) Protein Pak 125 size exclusion column. Purified proteins were dissolved to 1 mg/ml in PBS for use as native or boiled (10 min) antigen.

2.4.3. Milk antigens

Non-fat dry milk standard reference material, NIST SRM#1549 was dissolved in PBS as supplied for use as antigen. Purified caseins (α , β and κ isoforms, Signalchem, Richmond BC, Canada) and β -lactoglobulin (BLG, Sigma-Aldrich) were dissolved in PBS for use as native antigen. Purified proteins were boiled for 1 h in water bath or denatured by treating with 1% SDS plus 10 mM DTT for 5 min at 75 °C (Watanabe et al., 2005).

2.5. Polyclonal antibodies and human serum

Rabbit polyclonal antibodies against allergenic proteins (Table 2) were produced at FDA, Center for Food Safety and Applied Nutrition,

Laurel, MD, USA in an AAALAC-accredited facility. All rabbit injections consisted of 100 µg of purified antigen. Briefly, rabbits were inoculated on day 0 with antigen suspended in 50% complete Freund's adjuvant in normal saline, 50% incomplete Freund's adjuvant in normal saline at day 21 and in normal saline on days 35 and 50. A serum sample was collected prior to inoculation to use as a negative control. Rabbit antibodies that were generated against purified alpha casein protein (full length) and full length purified BLG protein (bovine source) was purchased from Bioorbyt LLC (San Francisco, CA, USA) and Genetex Inc. (Irvine, CA, USA), respectively. Human serum samples were obtained from Plasmalab International (Everett, WA, USA). Individuals having high concentrations of allergen-specific IgE in the serum by ImmunoCap® analyses (Pharmacia Diagnostics, Portage, MI, USA) were used (Table 3).

2.6. SDS polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot analysis

Twenty-five micrograms of protein per lane was separated using precast NuPAGE® Bis-Tris 4–12% gels (Life Technologies, Grand Island, NY, USA) under denaturing conditions. Proteins were transferred to nitrocellulose membranes (Whatman GmbH, Dassel, Germany) or Immobilon-FL membranes (EMD Millipore Corporation, Billerica, MA, USA). Membranes were blocked with Odyssey blocking solution (Li-Cor Biosciences, Lincoln, NE, USA) for analysis with polyclonal antibodies. For blots with human sera, membranes were blocked with the following solutions: for peanut allergic sera, 0.02 M Tris, 0.5 M sodium chloride (Tris-saline, pH 7.5) containing 1% bovine serum albumin (BSA) and 0.05% Tween 20; for egg allergic sera, Tris-saline containing 5% skim milk powder (SMP) and 0.1% Tween; and, for milk allergic sera, 5X signalLOCK™ blocking solution (KPL, Gaithersburg, MD, USA) plus 0.1% Tween-20. After blocking, membranes were incubated overnight with rabbit primary antibodies (1:1000 to 1:5000) or human serum (1:10) diluted in blocking solution. Secondary antibodies were goat anti-rabbit IRDye 680LT (1:10,000) from LI-COR Biosciences (Lincoln, NE, USA) and alkaline phosphatase (AKP)-conjugated mouse monoclonal anti-human IgE (1:1000) from BD-Pharmingen (San Jose, CA, USA). Proteins were visualised with an Odyssey® Infrared Imaging System (Li-Cor Biosciences) or BCIP/NBT phosphatase substrate (KPL). Peanut mixed standards for SDS–PAGE and Western blot were prepared by mixing 1.0 µg each of purified Ara h 1, Ara h 2, Ara h 3, and Ara h 6 proteins. Egg mixed standards consisted of 1.0 µg each of purified ovalbumin, ovomucoid, and ovotransferrin (Sigma–Aldrich) proteins and milk mixed standards consisted of 1.0 µg each of purified caseins (Signal-chem, Richmond, BC) and BLG (Sigma–Aldrich). Molecular weight standards were purchased from Li-Cor Biosciences (Lincoln, NE, USA) or Life Technologies (Grand Island, NY, USA).

3. Results and discussion

3.1. Effect of buffer on the extraction efficiency of protein from tempered chocolate

To investigate the effect of tempering or buffer components on the extraction efficiency of peanut, egg and milk proteins from a

dark chocolate matrix, samples were extracted with five different buffers. Table 1 shows the relative yield of total protein from untempered and tempered samples. Total protein content reflects the total allergenic and non-allergenic proteins in each sample. The protein extraction efficiency of HSB, PBS, and Tris buffers was lower than that of HSB/SDS/2-ME and carbonate buffers. Comparable amounts of total protein were extracted from untempered and tempered chocolate samples except when using the HSB/SDS/ME buffer, where total protein extracted from tempered samples exceeded that of untempered samples, although this difference is not significant ($p > 0.05$). The solubility and extractability of the proteins from untempered and tempered samples were improved by the high pH of the carbonate buffer and the addition of denaturing or reducing agents, such as SDS and 2-ME, to high salt buffer.

Two key steps for analysis of food allergens from complex processed food matrices are efficient extraction of allergenic proteins from food commodities and detection of the extracted target proteins with a proper qualitative and/or quantitative method (Faeste, Løvberg, Lindvik, & Egaas, 2007). Extraction represents a major cause of inaccurate and imprecise analytical results (Khuda et al., 2012). The yield of extracted allergenic protein depends on the matrix components, extraction buffer components, food texture, food processing procedures, properties of allergenic proteins and specific extraction procedures, including time and temperature. The extensive variation in matrix components and processing procedures of consumable foods poses a significant challenge in identification of a universal extraction solution that can optimally extract all allergenic proteins from all processed food commodities.

Studies with defatted and non-defatted peanut flour showed that the fat component of peanut flour did not affect the yield of peanut proteins with various buffers, but the yield of extracted peanut proteins were decreased upon light and dark roasting of peanuts as compared to raw peanuts (Rudolf, Ansari, Kern, Ludwig, & Baumgartner, 2012). Poms, Capelletti, and Anklam (2004) showed that elevated roasting temperature reduced yield of extracted protein from oil and dry roasted peanut by 50–75% and 75–80%, respectively, as compared to the raw material. In studies using heat processed model foods and allergenic material without matrix it was found that by increasing the processing temperature, the yield of extractable egg allergenic proteins decreased (Faeste et al., 2007; Fu, Maks, & Banaszewski, 2010). The yield of milk allergenic proteins was also affected by food processing and matrix components (Monaci, Brohée, Tregoeat, & van Hengel, 2011; Steinhoff et al., 2011). As with our results, previous comparisons of extraction solutions showed that buffers containing a surfactant, such as Tween 20, Triton X-100 or SDS, high salt and 2-ME improved the isolation of allergenic proteins from complex heat-processed food matrices (Faeste et al., 2007; Steinhoff et al., 2011; Watanabe et al., 2005; Westphal et al., 2004).

3.2. Processing effects on the extraction and detection of specific and clinically relevant peanut, egg, and milk allergenic proteins from incurred dark chocolate

Although certain buffers may be preferable for extraction of total protein from food samples, specific food allergenic proteins

Table 1
Protein content of untempered and tempered dark chocolate samples extracted with (A) HSB, (B) HSB/SDS/ME, (C) Carbonate, (D) Tris and (E) PBS buffers. Protein levels are expressed as mean ± standard deviation.

Chocolate samples	Total protein concentration (mg/ml) of indicated extractants				
	HSB pH = 7.4	HSB/SDS/ME pH = 7.4	Carbonate pH = 9.5	Tris pH = 8	PBS pH = 7.4
Untempered 0 ppm	0.08 ± 0.00	5.1 ± 0.57	2.26 ± 0.33	0.163 ± 0.05	0.116 ± 0.06
Untempered 500 ppm	0.107 ± 0.03	5.56 ± 0.67	2.89 ± 0.06	0.118 ± .02	0.153 ± 0.02
Tempered 500 ppm	0.078 ± 0.01	6.67 ± 1.19	1.98 ± 0.22	0.169 ± 0.03	0.157 ± 0.06

may not be efficiently extracted using the same buffers. Allergen-specific immunochemical staining was performed for qualitative characterisation of allergenic proteins extracted from untempered and tempered chocolate samples using each of the five buffers. Processing-induced modifications of allergenic proteins can either decrease or increase immunoreactivity of specific allergens. Most available immunodetection methods use antibodies raised against one or several unmodified proteins for food allergen detection. To overcome the issues related to the detection of food allergens in processed complex food matrices and for more robust recognition, antibodies against processed peanut, egg, and milk allergens (Table 2), in addition to native proteins, were generated and used for Western blot analysis. Human sera (Table 3) were also utilised to determine extraction efficiency of clinically relevant allergenic proteins.

3.2.1. Peanut

An optimum combination of extraction procedure and antibody for immunodetection of specific peanut allergenic proteins from processed foods has not been established (Pomes, Vinton, & Chapman, 2004; Rudolf et al., 2012; Westphal et al., 2004).

Table 2

Rabbit antibodies used for the analysis of peanut, egg, and milk allergens in chocolate sample extracts.

Allergen	Specificity of rabbit antibodies
Peanut allergens	Raw peanut Ara h1
	Light roasted peanut Ara h2
	Light roasted peanut Ara h3
	Light roasted peanut Ara h6
	Denatured raw peanut
	Light roasted peanut
	Raw peanut
Egg allergens	Raw egg white
	Boiled egg white
	Fried egg white
	Native ovomucoid
	Boiled ovomucoid
	Boiled ovalbumin
Milk allergens	Raw milk
	Native alpha (a) casein
	Boiled casein
	Denatured casein
	Native BLG
	Boiled BLG

Table 3

Patient sera used for immunoblot analysis of chocolate extracts. Allergen-specific IgE levels of peanut, egg, milk-allergic and non-allergic patients were determined using ImmunoCAP®.

Patient	IgE kU/L
Peanut-allergic (a)	>100
Peanut-allergic (b)	>100
Peanut-allergic (c)	>100
Egg-allergic (a) ¹	78
Egg-allergic (b)	30.5
Egg-allergic (c)	35.1
Egg-allergic (d)	22
Egg-allergic (e)	22.5
Egg-allergic (f)	88.6
Milk-allergic (a)	86
Milk-allergic (b) ¹	78
Milk-allergic (c)	>100
Non-allergic (a)	Negative
Non-allergic (b)	Negative
Non-allergic (c)	Negative

¹ This patient is allergic to both egg and milk.

Fig. 1A shows specific recognition of peanut proteins in five different chocolate sample extracts as detected with rabbit polyclonal antibodies having specificities listed in Table 2. Using antibody specific for native Ara h 1, this protein was detected weakly in most chocolate extracts, and it appears that this antibody is also weakly cross-reactive with other extracted proteins. Ara h 1 is known to become rapidly denatured and/or aggregated following various processing conditions, including heat (Koppelman, Bruijnzeel-Koomen, Hessing, & de Jongh, 1999), which also hampered our ability to produce sufficiently purified Ara h 1 from roasted peanut for antibody production. These processing-induced structural changes could result in reduced affinity of the anti-Ara h 1 antibody, since Ara h 1 purified from unroasted peanut, which was used in the mixed standards, was readily recognised by this antibody. Anti-Ara h 2 detected distinct Ara h 2 dimers in HSB/SDS/2-ME extracts of untempered and tempered samples, demonstrating that Ara h 2 proteins are optimally extracted under reducing and denaturing conditions. Anti-Ara h 3 recognised Ara h 3 protein in the carbonate and HSB/SDS/ME extracts. Ara h 6 protein was visualised with the autologous anti-Ara h 6 and anti-light roasted peanut antiserum in the HSB/SDS/2-ME extract. Faint bands of Ara h 1 protein were visualised with antibody against denatured peanut in the HSB/SDS/2-ME extract. No peanut allergens were visualised by Western blot when an antibody raised against whole raw peanut flour was used (not shown). In general, staining intensity was lower in extracts of tempered chocolate as compared to untempered when extracted with the same buffer. These observations led to the conclusion that the addition of denaturant and reducing agent in the extraction buffer improved the extraction efficiency of most peanut proteins as visualised by immunochemical staining with polyclonal antibodies. This is probably due to the increased solubility of peanut proteins by the 2-ME cleavage of disulphide bonds and/or denaturation with SDS.

In general, stronger signals were observed with Ara h 2 and Ara h 6 proteins as compared to Ara h 1 and Ara h 3 proteins, when using autologous rabbit antisera, indicating that these may be preferable target proteins for immunodetection. Interestingly, these proteins have been identified as the major peanut allergens, based upon assays of allergen effector activity (Chen, Wang, El-Mezayen, Zhuang, & Dreskin, 2012; Zhuang & Dreskin, 2012).

Western blot detection of peanut proteins using human allergic sera is shown in Fig. 1B. Most sera had a similar pattern of reactivity, and processing did not appear to reduce signal strength, as observed previously (Koch et al., 2003). IgE reactivity was best observed using the carbonate extracts. Most of the test buffers were able to extract one or more peanut IgE-binding proteins, although with a low signal strength, in both untempered and tempered chocolate samples. This is despite the fact that peanut allergic sera had very high IgE levels by ImmunoCAP®. No reactivity was observed using a non-allergic human serum. The low signal could be due to matrix-induced structural modifications and/or alteration of epitopes by the reducing conditions of the SDS-PAGE. More allergic sera will have to be tested to draw definitive conclusions. Previous studies have also shown that the level of IgE binding to specific peanut proteins by Western blot does not necessarily correlate with ImmunoCAP® levels, nor does it necessarily reflect the severity of a clinical allergic response (Clarke et al., 1998; Hourihane, Kilburn, Dean, & Warner, 1997).

3.2.2. Egg

We focused on the egg proteins ovalbumin and ovomucoid, which are the immunodominant allergens in egg white (Mine & Yang, 2008). Ovomucoid is considered the major allergen in processed food because it is more resistant to heating and digestion than ovalbumin (Benedé, López-Fandiño, Reche, Molina, & López-Expósito, 2013; Haneda et al., 2012). To detect modified egg

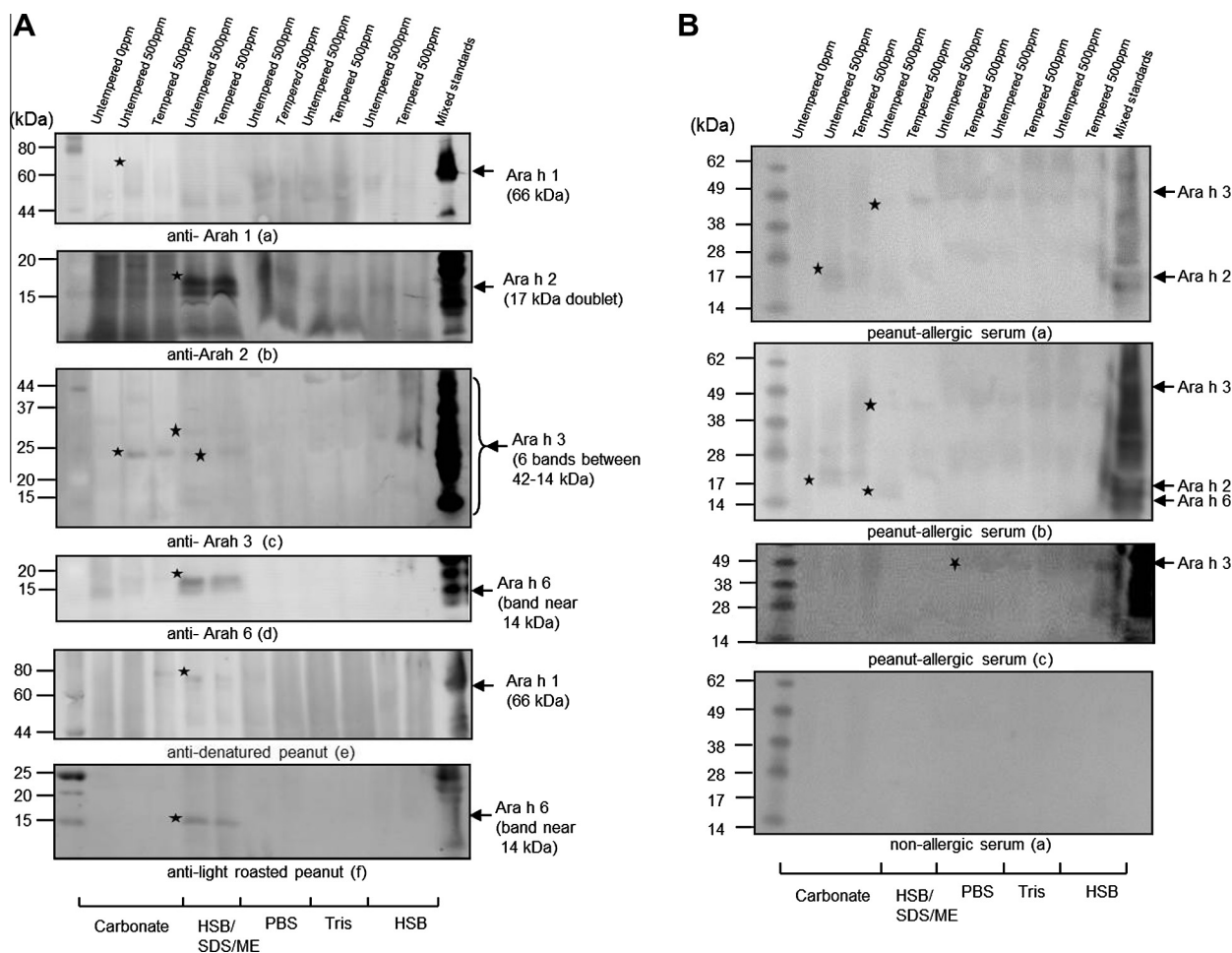


Fig. 1. (A) Identification of peanut allergenic proteins in extracts of untempered and tempered chocolate using polyclonal antibodies against (a) raw peanut Ara h 1, (b) light roasted peanut Ara h 2, (c) light roasted peanut Ara h 3, (d) light roasted peanut Ara h 6, (e) denatured raw peanut, and (f) light roasted peanut. (*) Indicates the protein recognised by the antibody. (B) Extracts of untempered and tempered chocolate were also evaluated by immunoblot using sera from peanut allergic patients. Specific allergenic proteins recognised are indicated by an arrow to the right of the immunoblot.

allergens from tempered chocolate samples, several common food processing strategies were utilised for preparing ovalbumin and ovomucoid antigens for generation of polyclonal antibodies. Immunoblot analysis of egg protein buffer extracts from chocolate samples with antibodies produced against processed and unprocessed egg antigens are shown in Fig. 2A. Ovalbumin and ovomucoid were recognised with antibodies against raw egg white and fried egg white in all buffer extracts except HSB/SDS/ME, but signal strength was lower in the tempered samples. The weak binding of antibodies against native or raw antigens and purified egg proteins in both the untempered and tempered samples extracted with buffer containing SDS and 2ME was probably due to the alteration of the antibody-reactive sites of egg allergens by SDS or 2ME (Watanabe et al., 2005). Antibodies against boiled egg white showed high affinity for these proteins in all extracts, but staining intensity was visibly lower in extracts of tempered chocolate. Antibodies produced against native ovomucoid detected this protein with greater intensity in HSB and Tris extracts, but with reduced intensity in tempered samples. Antibodies against boiled ovomucoid and boiled ovalbumin detected proteins in all extracts. These blots demonstrate that antibodies prepared against boiled egg white, boiled ovomucoid, and boiled ovalbumin had greater affinity for extracted egg proteins in tempered and untempered chocolate extracts using most buffers, whereas egg proteins were more weakly detected when raw proteins were used for antibody production. Immunoblot analysis (Fig. 2B) with human sera from

egg allergic patients showed differences in detection of egg allergenic proteins depending on the extraction buffer utilised and, in one case, serum reacted with extracted ovotransferrin. Almost no reactivity was observed with carbonate buffer extracts. These results show that antigenic egg proteins were optimally extracted with PBS, Tris, and HSB buffers from tempered chocolate even though the yield of total protein was higher using a buffer containing SDS and 2-ME (Table 1). It was previously observed that dry heat treatment significantly reduces the binding of rabbit antiserum (IgG) and human allergic sera (IgE) to ovalbumin, while heat treatment negatively affected the binding of human IgE to ovomucoid with little impact on binding to rabbit IgG (Jiménez-Saiz, Belloque, Molina, & López-Fandiño, 2011). We show that use of detecting antibodies generated against heat treated egg proteins can improve detection of processed egg allergenic proteins in this matrix, but, unlike the situation with peanut allergens, extraction of egg allergens from this matrix was not improved using extraction buffer with added SDS and 2-ME.

3.2.3. Milk (casein and BLG)

The influence of different extraction buffers on the extractability and immunoreactivity of casein and BLG from untempered and tempered chocolate samples are shown in Fig. 3A. Antibodies against native casein and BLG, as well as in-house produced antibodies against NIST SRM#1549, boiled and denatured casein and boiled BLG were compared in their ability to bind milk allergens

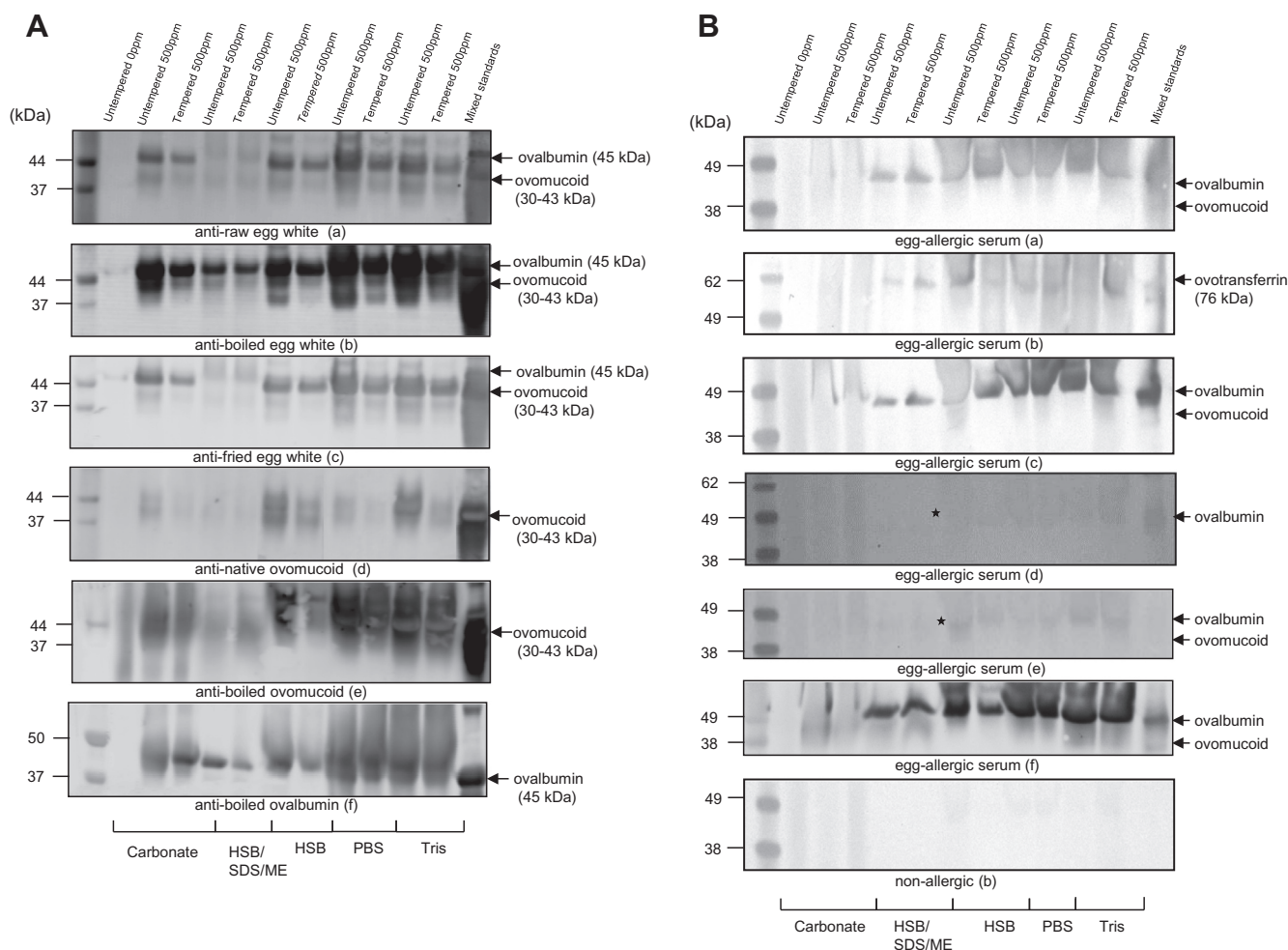


Fig. 2. (A) Identification of egg allergenic proteins in extracts of untempered and tempered chocolate using polyclonal antibodies generated against (a) egg white raw, (b) egg white boiled, (c) egg white fried, (d) native ovomucoid, (e) boiled ovomucoid, and (f) boiled ovalbumin. (B) Extracts of untempered and tempered chocolate were also evaluated by immunoblot using sera from egg allergic individuals. (*) Indicates the protein recognised by the sera.

from processed and unprocessed dark chocolate following extraction with various buffers. In general, antibodies produced against processed antigens bound a greater number of proteins and with greater intensity as compared to antibodies against native antigens. Also, binding to proteins in most extracts of tempered chocolate were lower or negative using antibodies to native casein or native BLG. Interestingly, casein proteins extracted with SDS and 2-ME were readily visualised with antibody against boiled casein but only weakly recognised by antibodies generated against denatured or native casein. These results show that carbonate, PBS, Tris and HSB buffers may be more suitable for casein extraction despite the decreased signal in the tempered chocolate extracts. Antibodies against native BLG reacted with BLG protein in all except the Tris buffer extracts, where very low or no signal was observed. Using antibodies against boiled BLG resulted in an increased signal in most extracts. Both antibodies generated against BLG gave a weaker signal using tempered chocolate extracts. In contrast to casein proteins, SDS and 2-ME in the extractant improved extractability/solubility of BLG proteins.

The binding patterns of IgE to milk proteins using human allergic sera differed among milk-allergic individuals (Fig. 3B). Milk allergic serum (a) recognised both casein and BLG proteins in all test extracts with varying intensity, whereas serum (b) reacted with casein protein mostly in carbonate extract and BLG protein was observed only in the HSB/SDS/ME extract. Milk allergic serum (c) was reactive with casein protein in all test extracts, but with

different signal intensities. No protein band was visualised in any test extracts with non-allergic sera. These results show that the extraction buffers did not consistently influence the IgE binding to casein and BLG proteins by Western blot, but tempering negatively impacted the binding of human IgE in some cases.

Casein and BLG are major milk allergens. As a consequence of food processing effects on the antigenic determinants of milk allergenic proteins, their recognition by antibodies used for immunodetection in processed foods may be impaired. Most food processing procedures decrease solubility, extractability, and detection of milk allergenic proteins, especially BLG, thereby negatively impacting most quantification methods (Diaz-Amigo, 2010; Khuda et al., 2012; Monaci et al., 2011). Certain buffers can improve the extraction of milk proteins but optimum protein extraction also depends on the food matrix (Steinhoff et al., 2011). Casein is relatively thermostable, whereas BLG is thermolabile. However, the thermostability of BLG is improved following heat treatment in a food matrix, possibly through its interaction with casein micelles or coaggregation with α -lactoglobulin (Paschke & Besler, 2002; Ehn, Ekstrand, Bengtsson, & Ahlstedt, 2004; Wal, 2004).

4. Conclusions

Immunoassays are the most common analytical methods used for screening of food products for declared or undeclared food

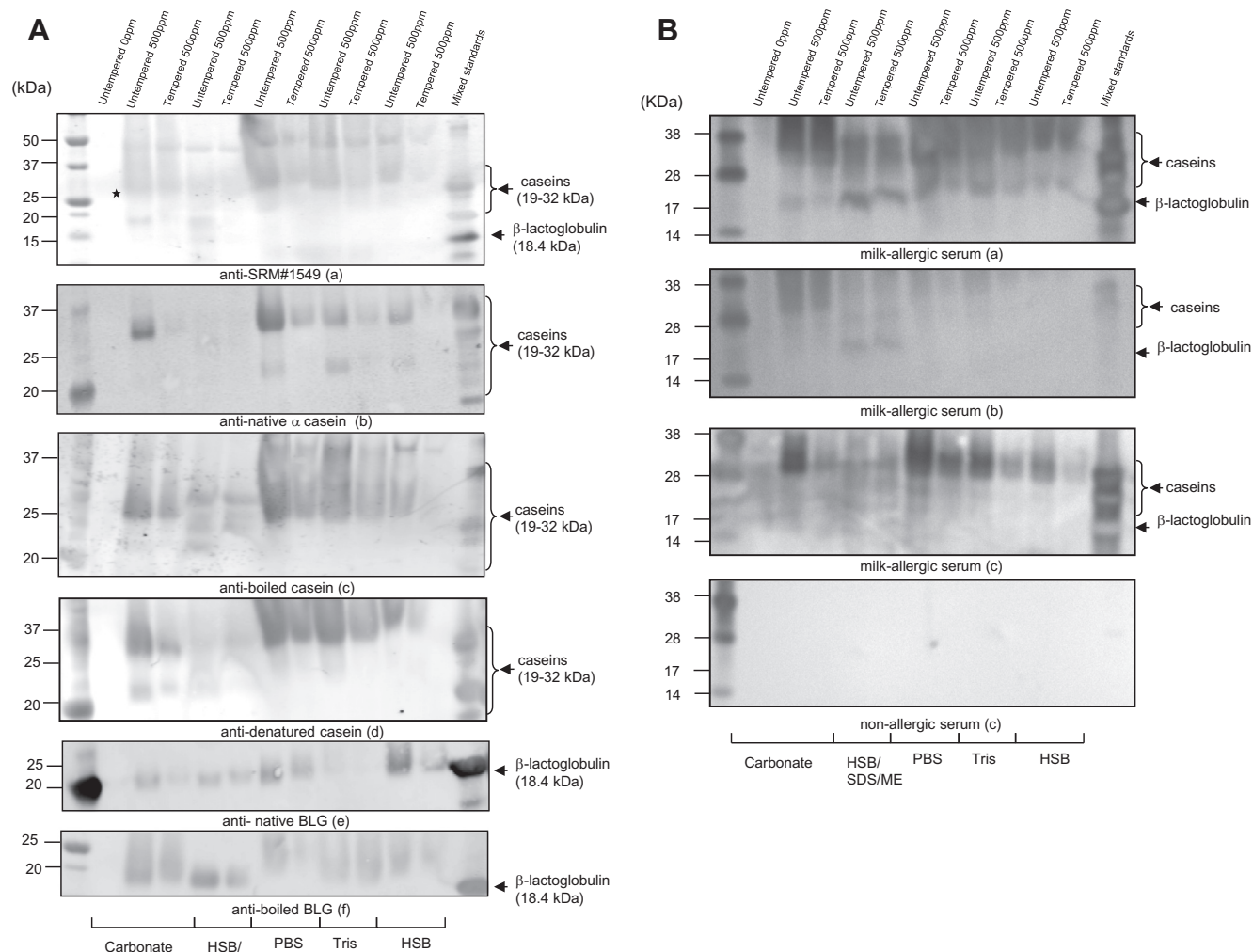


Fig. 3. (A) Identification of milk allergenic proteins in extracts of untempered and tempered chocolate using polyclonal antibodies generated against: (a) raw milk, (b) native α casein, (c) boiled casein, (d) denatured casein, (e) native BLG, and (f) boiled BLG. (B) Extracts of untempered and tempered chocolate were also evaluated by immunoblot using sera from milk allergic patients.

allergens due to their ease of use and sensitivity. The outcome of immunoassays from complex food matrices is affected by decreased solubility/extractability of food allergenic proteins and reduction/lack of antibody recognition of modified protein antigenic determinants, both of which can be a consequence of processing procedures. The detection of peanut, egg, and milk allergens by immunoassay is compromised when using processed samples in complex food matrices, such as chocolate (Khuda et al., 2012; Monaci et al., 2011; Pele et al., 2007; Westphal et al., 2004). By comparing qualitative protein recognition using rabbit antisera and human allergic sera, we have identified the optimum buffers for the extraction of specific allergenic proteins in this matrix. Additionally, we found that although some buffers were superior for the extraction of total protein from dark chocolate, this did not always translate to improved detection of allergenic proteins.

The optimum extraction of peanut proteins was obtained with buffer containing surfactant (SDS) and reducing agent (2-ME), and subsequent peanut detection was improved using rabbit antibodies against processed antigens, especially anti-Ara h 2 derived from roasted peanut. Clinically relevant peanut allergenic proteins were extracted with most of the test buffers, although detection was weak with the human allergic sera, and tempering did not

have an observable effect on IgE binding. PBS, Tris, and HSB were the most appropriate extractants for immunoreactive and clinically reactive egg allergens from tempered chocolate samples, and recognition was improved using antibodies generated against boiled egg allergens. Carbonate, PBS, Tris and HSB buffers were optimum for casein extraction and casein recognition by rabbit antisera were negatively impacted by tempering, whereas HSB/SDS/2-ME was the most suitable extractant for BLG extraction and immunoreactivity. Rabbit antisera against processed casein and BLG recognised more protein epitopes in the extracts than antibodies against the native proteins.

Optimum immunodetection of allergenic proteins from a processed chocolate matrix depended on the extraction buffer used, the characteristics of the individual protein, and the affinity/specificity of the detecting antibody. This work will help to understand and standardise critical immunoassay parameters for the improved analysis of food allergens from complex foods matrices.

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