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Comparison of extraction conditions for milk and hen's egg allergens

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Abstract

The evaluation of recovery rates by extracting dried milk and egg powder using eleven different extractants gave approximately similar results for both foods. Compared to the other extraction solutions investigated, "1% Tween 20 and 0.4% Triton X-100" and "4 % SDS" are the most suitable extractants to isolate proteins of hen's egg or milk. Comparing calculated protein recovery rates of egg and milk powder extracts the results clearly indicated that the choice of a suitable extractant is of particular importance. Qualitative investigation of the extracts via LDS - PAGE followed by silver staining as well as immunoblotting confirmed the results of protein quantification. Hence, the immunoblots showed that the extraction agents had no negative influence on the antigenicity of the extracted allergenic proteins. In this study, variation of extraction temperature neither led to any benefit in extraction quality nor to degradation. Changing pH did not reveal any trends, but progressive protein hydrolysis under strong alkaline conditions. Evaluation of recovery rates as well as results of unspecific and specific staining of the extracts showed that an extraction time of one hour is sufficient for an appropriate sample preparation. For investigations with and without food matrix different results were obtained. In summary, wheat starch did not influence the extraction quality within all examined materials and different extractants. In contrast, using fat powder and dry cake mix, respectively, led to different results in the extraction procedure. When fat powder and dry cake mix were used as food matrices, some protein recovery rates decreased and

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some increased depending on the allergen material. These results highlight the fact that the suitability of the extractant not only depends on the properties of the allergen but furthermore on the type of matrix containing the allergen.

Keywords: Food allergy, hidden allergens, extraction of allergens, hen's egg, cow's milk

Introduction

Cow's milk and hen's egg as well as their products belong to eight food or food groups which are responsible for about 90 % of allergic reactions (Hefle and Taylor, 2004). The consumption of small amounts can already cause life-threatening or lethal reactions (Vieluf et al., 2002). Nevertheless, allergic persons can unintentionally consume allergenic material if these are unexpectedly part of a food product e.g. because of food contamination / cross contact within the manufacturing process (Hefle and Taylor, 2004). The threshold doses for triggering symptoms after ingestion of the offending food are strongly dependent on the patient's individual susceptibility and the allergic potential of the particular food. Clinical data shows that exposure to even about 1 mg of the allergen can elicit adverse reactions up to anaphylactic shocks in individuals with IgE-mediated food allergies (Besler et al., 2001; Moneret-Vautrin and Kanny, 2004). The legislation on food labelling only concerns allergenic ingredients that are knowingly and deliberately added to food products.

The analytical proof of the presence of small amounts of allergens, must use a technique sensitive enough to specifically detect the allergens in those amounts that might trigger allergic reactions in sensitized individuals. The determining step in allergen detection is the isolation of the allergens from the food which can be influenced by numerous factors. The diversity of foods in terms of composition and texture as well may require appropriate

55 extraction methods. The extraction solution must provide efficient isolation of the allergenic
56 proteins from the sample into a liquid phase and minimize background effects due to non-
57 specific binding. In food many ingredients are available to participate in complex physical and
58 chemical reactions with the allergenic proteins (Davis et al., 2001). Especially during the
59 manufacturing process the matrix might cause covalent modification of proteins through food
60 processing. As well as antigenicity, the extractability of allergens can be decreased in these
61 reactions, they might be unchanged or even increased (Besler et al., 2001).

62
63 The aim of this study was to evaluate and compare extraction solutions for the isolation of
64 milk and egg allergens. For this purpose extractability of allergenic proteins of dried milk
65 powder, sweet whey, acid whey, whole egg, egg yolk and egg white with eleven different
66 extraction agents was examined. Extractants were selected by collecting recipes already
67 described in literature to isolate allergens. In addition, chosen extraction solutions represent
68 a huge diversity in pH, ionic strength, and detergent content. As extraction quality describing
69 parameters protein yield, the composition of the proteins as well as the antigen patterns were
70 determined. To evaluate, if food matrix influences the extractability of proteins, wheat starch,
71 fat powder and an experimental dry cake mix were employed in the extraction experiments.

72
73 **Materials and Methods**

74 *Reagents and materials*

75 Milk powder, dried sweet whey, dried acid whey, whole egg powder, powdered egg yolk and
76 powdered egg white were obtained from Kampffmeyer Food Innovation GmbH (Hamburg,
77 Germany). Powdered egg white was purchased from Sigma-Aldrich Chemie GmbH (Munich,
78 Germany). To obtain powdered egg yolk, liquid whole egg was separated mechanically and
79 lyophilized referring to homogenization. Commercially available wheat starch (protein-free,
80 Heinrich Klenk GmbH & Co. KG, Schwebheim, Germany), fat powder (protein-free coconut
81 oil, Kampffmeyer Food Innovation GmbH, Hamburg, Germany) and an experimental dry cake
82 mix were used as matrix. The dry cake mix contained sucrose (70.5 %, Kampffmeyer Food

Innovation GmbH, Hamburg, Germany), fat powder (17.7 %), wheat starch (9.8 %), sodium hydrogen carbonate (1.6 %, Merck Chemicals, Darmstadt, Germany) and sodium chloride (0.4 %, Merck Chemicals, Darmstadt, Germany).

Sheep anti-casein serum and biotinylated rabbit anti-sheep serum were purchased from antibodies-online GmbH (Aachen, Germany). Rabbit anti-whey and rabbit anti-ovalbumin sera were both acquired from Sigma-Aldrich Chemie GmbH (Munich, Germany). Biotinylated goat anti-rabbit antibodies were bought from Rockland (Gilbertsville, PA, USA) and streptavidin-horseradish peroxidase was obtained from Bio-Rad Laboratories GmbH (Munich, Germany). All other reagents were of analytical grade and purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany), Fluka (Buchs, Switzerland) and Serva Electrophoresis GmbH (Heidelberg, Germany).

Composition of extractants

In this investigation, eleven different extractants were used to extract protein from dairy and egg products: A: 0.01 M phosphate-buffered saline containing 10 mM Na_2HPO_4 , 1 mM KH_2PO_4 , 3 mM KCl and 140 mM NaCl, pH 7.4 (Schubert, 2003); B: 0.01 M phosphate-buffered saline containing 2 mM NaH_2PO_4 , 8 mM Na_2HPO_4 and 150 mM NaCl, pH 7.4 (Hefle and Lambrecht, 2004); C: 0.01 M phosphate-buffered saline containing 150 mM NaCl, 10 mM Na_2HPO_4 , pH 7.2 (Leduc et al., 1999); D: physiological saline containing 150 mM NaCl, pH 6.6 (Langeland, 1982); E: 1 % Tween 20[®], pH 6.0 (Fernandez et al., 1999); F : 8 M urea, pH 7.7 (Natarajan et al., 2005); G: 100 mM acetate buffer, pH 3.8 (Hirose et al., 2004); H: 86 mM NaCl, 30 mM NaHCO_3 , 0.4 % phenol, pH 8.2 (Coca, 1922); I: 1 % Tween 20[®], 0.4 % Triton X-100[®], 280 mM NaCl, 40 mM NaH_2PO_4 , pH 7.4 (Yeung et al., 2000); J: 4 % Sodium dodecyl sulphate, pH 8.5 (Kato et al., 2001a); K bidestilled water. All extraction solutions were prepared using bidestilled water and were stabilized with 0.02 % NaN_3 . For investigations with the aim to optimize pH-value of selected extractants, J (pH 8.5) and I (pH

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3 110 7.4) were additionally prepared with pH-values adjusted to 10.5 and 12.5 and 9.4 and 11.4,
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5 111 respectively. Adjustment of pH was carried out with 0.1 M NaOH.
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9
10 113 *Protein isolation*

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12 114 Principally, extraction procedure was carried out as follows. According to the protein content
13
14 115 measured via Kjeldahl method, 30 mg milk powder, 100 mg sweet whey, 100 mg acid whey,
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16 116 25 mg whole egg powder, 15 mg dried egg white and 30 mg dried egg yolk, respectively,
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18 117 were weighed into a 50 mL polypropylene centrifuge tube (Nunc GmbH & Co. KG,
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20 118 Wiesbaden, Germany). In the case of matrix addition, 1 g matrix (wheat starch, fat powder,
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22 119 dry cake mix) was also weight in the centrifuge tube and the dry material was mixed 5 sec
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24 120 with a vortex mixer (VWR international, Darmstadt, Germany; mixing frequency 2,500 rpm)
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26 121 before extractant was added. Protein extraction was carried out by suspending sample
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28 122 material with 20 mL of extraction solution. The homogenate was extracted over night on a
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30 123 laboratory shaker (Bühler, Tübingen, Germany; shaking frequency 150 rpm) at room
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32 124 temperature and centrifuged afterwards 30 min at 10,000 rpm (Sigma-Aldrich Chemie
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34 125 GmbH, Munich, Germany). Solids were removed by filtration (Macherey-Nagel GmbH & Co.
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36 126 KG, Düren, Germany; filter paper grade MN 616). Investigations with the aim to optimize
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38 127 extraction temperature were additionally carried out at 4 °C on a laboratory shaker in a
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40 128 standard refrigerator (Linde, München, Germany) and at 40 °C using a laboratory incubator
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42 129 (IRC-1-U Clim-o-shake, Adolf Kühner AG, Birsfelden, Swiss; shaking frequency 150 rpm).
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44 130 For examination of extraction duration, protein isolation as described above was interrupted
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46 131 after 0.5, 1, 2, 4 and 8 hours. Each extraction process was performed in triplicate.
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53 133 *Protein quantification*

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55 134 Absolute protein content of used dry materials was determined via Kjeldahl method analysing
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57 135 the nitrogen content. The conversion factors from nitrogen to protein were as recommended
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59 136 6.38 (dairy products) and 6.25 (egg products), respectively. The protein concentration of the
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137 extracts was measured photometrically according to the method of Lowry (1951) using

bovine serum albumin (BSA) and ovalbumin respectively as standards. Photometrical measurement of protein content was favoured over Kjeldahl method because in comparison the latter is of high expenditure of time and technical effort. The Folin's reagent used in the Lowry method consists amongst others of phosphomolybdic / phosphotungstic acid. A standard curve was produced for each run using a 500 mg / L protein solution prepared using the appropriate extractant. The protein content of the extracts prepared with "Coca's solution" (containing phenol) was measured by the method of Bradford (1976) because phenol interferes with the Lowry method. Bradford reagent consists of Coomassie Brilliant Blue G-250 and phosphoric acid. Quantitative assessments of extracts and standards were performed by measuring optical density at 720 nm (Lowry method) and 590 nm (Bradford method) using a spectrometer (MRX Plate Reader, Dynex Technologies, Frankfurt, Germany).

Lithium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (LDS - PAGE)

Proteins were separated with precast NuPAGE® Bis-Tris 12 % gels (Invitrogen GmbH, Karlsruhe, Germany) under denaturing conditions at 200 V for 55 min using NuPAGE® MOPS [3-(N-morpholino) propanesulfonic acid] SDS running buffer (Invitrogen GmbH, Karlsruhe, Germany). Following the manufacturers instructions obtained extracts were diluted 1:1 in LDS (lithium dodecyl sulphate) sample buffer (Invitrogen GmbH, Karlsruhe, Germany) containing dithiothreitol for reduction at room temperature. Sample load was 20 µL per lane. Proteins were further silver stained or transferred by blotting method onto a nitrocellulose membrane.

Silver staining

The proteins were detected by standard silver staining procedure according to (Heukeshoven and Dernick, 1986) following LDS - PAGE. After fixing the gel (30 % ethanol, 10 % acetic acid in water; 30 min) it was sensitized in 0.1 % sodium thiosulfate (0.1 % Na₂S₂O₃, 30 % ethanol, 6.8 % sodium acetate in water). After washing with water the gel was incubated for 20 min in 0.2% silver nitrate solution (0.2 % AgNO₃, 0.02% formaldehyde in water).

Afterwards the gel was developed in 2.5 % sodium carbonate (2.5 % Na_2CO_3 , 0.01% formaldehyde in water). Staining was terminated using 1.8 % EDTA-solution (1.8 % ethylenediaminetetraacetate in water).

Immunoblotting

The isolated proteins separated by LDS - PAGE were transferred electrophoretically from the gel onto the surface of a nitrocellulose membrane (2 μm , Whatman, Kent, UK) using semidry blotting method. In brief, dried membranes were blocked with blocking solution (containing 0.5 % Tween 20[®], 0.9 % sodium chloride and 0.6 % tris base) and incubated overnight with sheep anti-casein (1 : 400), rabbit anti-whey (1 : 200000) and rabbit anti-ovalbumin (1 : 200000) antibodies, respectively, at room temperature. Membranes were then incubated consecutively with biotinylated rabbit anti-sheep serum (1 : 100000; milk powder) or biotinylated goat anti-rabbit serum (1 : 7500; powdered sweet whey, powdered acid whey, whole egg powder, powdered egg yolk, powdered egg white) and streptavidin - horseradish peroxidase. Staining solution containing TMB and DONS (0.06 % 3, 3', 5, 5' - Tetramethylbenzidine, 0.2 % dioctylsodiumsulphosuccinate, 25 % ethanol, 0.7 % citric acid, 1 % Na_2HPO_4 in water) was used for staining.

Results

Protein quantification

Protein recovery rates in the extracts were estimated by relating theoretical calculated protein content measured via the Kjeldahl method to total protein yield of the extract measured with Lowry or Bradford method (data not shown). The estimated recovery rates are presented in Table 1 and Table 2. Extracting milk proteins from milk powder, recovery rates range from 69 to 109 % except for solution G yielding 16 % protein. Highest recoveries in protein isolation were achieved using solution I and J as extractants. Estimated recovery rates for extraction of milk proteins from milk powder in dry cake mix ranged from 40 to 87 % except for solution G recovering 14 % protein. Within these extracts, extractants B, D and J gave the highest protein recoveries. Extracting proteins from milk powder in wheat starch

and fat powder, the highest protein contents were obtained in the extracts prepared using solution I, J and C or I and D, respectively (Table 1).

For all the egg materials which were examined, the extraction agents I and J aqueous solution gave the highest protein yields (Table 2). In contrast to the extractants mentioned above, the protein contents of the extracts prepared using saline solutions (A, B, C, D), G or H to extract egg proteins from egg material led to recovery rates lower than 30 %. Where egg proteins were isolated from egg powder in dry cake mix, solutions I and J gave the highest protein recovery rates. Extracting proteins from egg material in wheat starch and fat powder as food matrix, the highest protein contents were measured in the extracts prepared using solutions I and J. But also E achieved high recovery of protein from egg material in wheat starch (Table 2).

In this study, optimization of pH-value of extractants as well as extraction temperature was carried out. Therefore, solution I (pH 7.4) and J (pH 8.5) were additionally prepared having a pH of 9.4 and 11.4 (I) as well as 10.5 and 12.5 (J), respectively. Extraction procedures were simultaneously performed at three different extraction temperatures (4 °C, RT = room temperature and 40 °C). The estimated recovery rates are presented in Tables 3 and 4. Comparing the protein yields there is not much of a difference. The deviation of the obtained recovery rates is of the order of the calculated standard deviation.

With the aim to investigate optimum extraction duration, extraction procedures were performed at different extraction temperatures using solution I (pH 7.4) and J (pH 8.5) as extractants to isolate milk and egg proteins from milk powder and whole egg powder and from wheat starch and cake mix, respectively. Extraction procedures were stopped after 30 min, 1 h, 2 h, 4 h and 8 h and protein contents were measured subsequently. Resulting recovery rates are shown in Tables 5 and 6. Regardless of extractant and extraction

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3 223 temperature there is not much of a difference comparing protein recoveries. The extracts
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5 224 prepared of solution J have slightly higher protein contents than those made of by solution I.
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10 226 *Lithium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (LDS - PAGE)*
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12 227 The composition of the isolated protein extracts were analyzed by electrophoretic separation
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14 228 and silver staining. Comparison of (i) the number of protein bands and (ii) the intensities of
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16 229 characteristic protein bands illustrates considerable protein diversity between the examined
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18 230 extracts. LDS - PAGE and silver staining patterns of proteins extracted from egg powder and
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20 231 milk powder using different extractants are shown in Figure 1. Those extracts prepared from
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22 232 egg powder using solution I, J and E gave particularly strong stained bands of conalbumine,
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24 233 ovalbumine and ovomucoid. Extracts prepared using solution G or H gave weakly stained
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26 234 bands of characteristic egg proteins. Similar results were obtained extracting powdered dairy
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28 235 products. The extractants I, J and E gave strongly stained bands of characteristic milk
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30 236 proteins α -lactalbumin, β -lactoglobulin and casein, whilst solution G gave weakly detected
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32 237 bands representing characteristic milk proteins. Analysis of protein patterns of extracts
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34 238 obtained extracting allergen material out of food matrix did not reveal wide differences in
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36 239 band intensity of characteristic milk and egg allergens.
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42 241 The influence of the pH-value of the extractants was investigated.. Solution I was prepared
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44 242 having pH-values of 7.4, 9.4 and 11.4, respectively. The pH-value of solution J was adjusted
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46 243 to 8.5, 10.5 and 12.5, respectively. Detection of the electrophoretically separated extracts via
47
48 244 silver staining demonstrated that extracted proteins out of egg products and milk products led
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50 245 to similar results. Figure 2 shows the LDS - PAGE and silver staining patterns of proteins,
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52 246 adjusted to different pH-values and used for extraction at 4 °C, room temperature and 40 °C.
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54 247 Comparing the band intensities of the characteristic proteins in the extracts prepared with
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56 248 solution I pH 7.4 and pH 9.4 no differences appeared. Likewise, the band intensities of the
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58 249 characteristic proteins in the extracts obtained using solution J pH 8.5 were as strong as
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60 250 those prepared at pH 10.5. Any characteristic proteins could be visualized in the extracts

made of solution J with a pH of 12.5. Conversely, any characteristic proteins detected after electrophoretic separation of the extracts made from solution I with a pH of 11.4 or the detection of the characteristic proteins was much weaker.

The band intensities obtained via silver staining of milk and egg proteins isolated at different temperatures were almost identical. Extracts obtained at the end of varied extraction durations using solution I (pH 7.4) and J (pH 8.5) were electrophoretically separated and proteins were detected via silver staining. Regardless of extractant and extracted material, band intensities of characteristic proteins did not differ in terms of extraction duration (data not shown).

Immunoblotting

Whether the estimated extractants ensure comprehensive isolation of intact allergens specific immunochemical staining on nitrocellulose membrane after LDS - PAGE and Western Blotting was performed (Figures 3 and 4). The extracts derived with solution I, J and E present most strongly stained bands of ovalbumine compared to solutions G and H in the immunoblots. The results of unspecific staining procedure as well as the determination of the protein concentration are reflected in the results of immunochemical staining. Rabbit anti-ovalbumin antibody used for detection of albumin also detects conalbumin (76 kDa) as characteristic egg protein. Quite similar results were obtained by immunochemical detection of milk proteins. Band intensities of milk proteins of purchased extracts looked rather similar to the extracts described in this paper and Immunoblots showed only slight variations in protein recovery rates. Anti-casein antibody used for detection of casein also detects bovine serum albumin (BSA; 76 kDa) as characteristic milk protein.

The influence of wheat starch and dry cake mix on extraction efficiency and quality was investigated by comparing the extract properties prepared with matrix addition and without as described above. Addition of 8 M urea as extractant to wheat starch or dry cake mix

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3 279 containing samples caused a strong enhancement of extract viscosity. Those solutions were
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5 280 unemployable for further analysis. Band intensities referring to silver and immunochemical
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7 281 staining do not differ noteworthy comparing the extracts made with matrix addition or without
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9 282 (Figures 3 and 4). Considering dilution factors, extracts of solution I, J as well as E present
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11 283 most strongly stained bands of ovalbumine compared to solutions G and H in the
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13 284 immunoblots. Purchased extracts showed similar band intensities. The immunoblots affirm
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15 285 the slight variation in protein recovery rates obtained for extracts made of dairy products.
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20 287 Comparing the band intensities of the characteristic proteins in the extracts prepared with
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22 288 solution I (pH 7.4 and pH 9.4) and J (pH 8.5 and 10.5), respectively, no differences
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24 289 appeared. Any characteristic proteins could be visualized in the extracts made of solution J
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26 290 pH 12.5 whilst the detection of the characteristic proteins extracted via solution I" pH 11.4
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28 291 was much weaker.
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33 293 The band intensities obtained via immunochemical staining of milk and egg proteins isolated
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35 294 at different temperatures were almost identical. Regardless of extractant and extracted
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37 295 material, the band intensity of the characteristic proteins did not differ in terms of extraction
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39 296 duration (data not shown).
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43
44 298 **Discussion**

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46 299 The ultimate prerequisite for sensitive analytical allergen detection is an efficient and reliable
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48 300 extraction method.
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51 301 *Protein recovery rates*

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53 302 Protein recovery rates were calculated by comparing the total protein concentration of the
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55 303 food sample with the protein yields of the extracts. The photometric detection, which was
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57 304 much more suitable for the screening of the extracts than the Kjeldahl procedure, only
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59 305 provides a relative but not an absolute quantification of the proteins. Nevertheless, the
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306 relative quantification was sufficient to assess the efficiency of the different extractants.

307
308 *Ranking the best extractant*
309 The evaluation of recovery rates of extracted milk and egg material using eleven different
310 solutions led to similar results. The solutions I and J achieved the highest protein yields.

311 In the case of milk samples, the extractants had only minor impact on the extraction
312 efficiency. On the other hand, the extraction efficiency of egg material was much more
313 sensitive to the solution used for the protein extraction. The major difference between milk
314 and egg is the fat content. Because of the higher fat content of egg material, extractants
315 containing a detergent yielded higher protein recovery rates as compared to those without.
316 Emulsifying properties of egg-lecithin alone are not sufficient to extract egg powder properly.
317 These results clearly indicate that the composition of the extractant is of particular
318 importance for the efficiency and quality of the extract.

320 *Quality of the protein extracts*

321 Analysis of the extracts by LDS - PAGE followed by silver staining as well as immunoblotting
322 confirmed the results of protein quantification. Solution I as well as J led to the strongest
323 bands of characteristic milk and egg proteins, respectively. However, it has to be considered,
324 that silver staining generally possesses a lower sensitivity than photometrical protein
325 quantification. Slight variations concerning protein content in the extracts cannot be
326 discriminated after electrophoretical separation.

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328 Detection of selected proteins by western blotting clearly demonstrated that both extraction
329 solutions had no negative influence onto the antigenicity of the extracted allergenic proteins.
330 Solution I and J are the most qualified extractants to isolate proteins of milk. In case of hen's
331 eggs, they are the only suitable solutions for the efficient extraction of proteins. Hence, hen's
332 egg is more sensitive to the extractant than milk.

334 *Optimization of the extraction procedure: temperature and pH*

For the elucidation of the optimal temperature range, extractions were carried out at 4 °C, room temperature and 40 °C. According to Howard et al. (1988) solubility of proteins can be increased at 40 °C compared to lower temperatures. In contrast, thermal treatment of allergens can influence conformational epitopes, which might lose their binding capacity to specific antibodies (Besler et al., 2001). In the worst case, false-negative results are obtained. In this study, variation of extraction temperature neither led to any benefit in extraction quality nor to degradation. Comparison of recovery rates obtained via extraction at different temperatures resulted in essentially similar results. Also unspecific staining as well as immunoblots did not show any differences in band intensity comparing the extracts prepared at different extraction temperature. The influence of pH-values of extractants was investigated using solution I and J prepared having different pH-values. Solubility of proteins strongly depends on the pH-value of the medium. Proteins have minimum solubility in water or salt solutions at the pH which corresponds to their isoelectric point (pI). Because milk proteins as well as egg proteins have pI lower than 7 (except lysozyme), solution I (pH 7.4) was additionally prepared having a pH of 9.4 and 11.4 and solution J (pH 8.5) was prepared having pH-values of 10.5 and 12.5. In this study, evaluation of the influence of the protein content in the extracts changing the pH did not show any tendencies. In contrast, analysis of unspecific staining of the electrophoretically separated extracts demonstrated, that solution I with a pH of 11.4 caused a partial hydrolysis of the proteins, whilst solution J with a pH of 12.5 brought the proteins about to a total hydrolysis. Alkaline hydrolysis of proteins causes progressive disintegration up to small peptides so that they are not detectable neither via unspecific nor specific staining procedure. As a consequence, these extractants are not applicable for further analysis damaging allergens antigenicity, although, solution I pH 7.4 and 9.4 as well as J pH 8.5 and 10.5 did not show this hydrolysis effect in the immunoblots.

359

360 *Optimization of the extraction duration*

361 To establish an extraction procedure in routine analyses, sample preparation should not be
362 too time-consuming. Otherwise, extraction duration has to guarantee maximum recovery of

proteins. Evaluation of recovery rates, results of unspecific and specific staining of the extracts showed that an extraction time of 1 hour is sufficient for an appropriate sample preparation.

Food matrix

Comparing the results of qualitative and quantitative investigations of extraction of milk and egg allergens out of certain dried milk and egg products with and without the presence of several other food matrices, different findings were obtained. In summary, wheat starch influences the extraction yield and the extraction quality with regard to the protein and antigen pattern for all materials which were tested and for different extractants. In contrast, using fat powder and dry cake mix, respectively, different extraction procedures led to different results. Whilst fat powder did not influence recovery rates of milk powder, for sweet whey and whole egg powder, up to twice as much protein was isolated from powdered egg yolk. However, isolation of egg proteins from egg yolk combined with dry cake mix resulted in protein recovery rates quite similar as those obtained for extracts without any matrix. On the other hand extraction of proteins from whey as well as powdered egg white blended with fat powder and dry cake mix, respectively, for some extractants lower protein recovery rates were found. Detection of electrophoretically separated proteins confirmed the results of protein quantification in giving weaker stained bands. These results highlight the fact that the suitability of extractant not only depends on allergen properties but furthermore on the surrounding food matrix.

What is the best procedure?

In this study, in the case of isolating proteins out of milk material "1% Tween 20 and 0.4% Triton X-100" pH 7.4 was the most suitable extraction agent. To extract egg proteins out of egg material the best results were obtained using 4 % SDS aqueous solution at pH 8.5. If the allergens are to be extracted out of complex food matrices containing sugars, fat and proteins, the extractant mentioned above are still suitable, but partly less quantitative. In the

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3 391 case of egg allergens in a starch and fat containing matrix Tween 20[®] as extractant gave
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5 392 high protein recoveries. Regardless of allergen material and food matrix, carrying out the
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7 393 extraction procedure for 1 hour at room temperature guarantees high protein yields without
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9 394 any negative influence on the allergens antigenicity.
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14 396 **Acknowledgments**

15
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20 399 Schwarz for technical support.
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Table 1: Protein recovery rates (%) estimated using different extractants to isolate milk proteins out of milk material or out of milk material in matrix

(WM = without matrix; WS = wheat starch; FP = fat powder; CM = cake mix)

Milk material	Matrix	Extractant										
		A	B	C	D	E	F	G	H	I	J	K
milk powder	WM	88 ± 4	85 ± 3	90 ± 2	89 ± 3	85 ± 2	86 ± 3	16 ± 1	69 ± 4	109 ± 4	94 ± 4	86 ± 8
	WS	87 ± 1	91 ± 4	92 ± 4	88 ± 4	79 ± 7	-	10 ± 0	69 ± 4	116 ± 6	92 ± 5	89 ± 2
	FP	86 ± 3	79 ± 5	83 ± 5	98 ± 5	44 ± 0	-	27 ± 5	78 ± 2	106 ± 4	85 ± 5	88 ± 2
	CM	71 ± 4	82 ± 9	72 ± 13	87 ± 13	40 ± 3	-	14 ± 0	81 ± 3	74 ± 5	82 ± 8	74 ± 4
Sweet whey	WM	81 ± 1	85 ± 3	91 ± 3	92 ± 3	85 ± 3	97 ± 4	53 ± 1	37 ± 1	138 ± 12	112 ± 3	85 ± 4
	WS	81 ± 4	89 ± 7	90 ± 1	93 ± 1	89 ± 6	-	45 ± 3	36 ± 1	142 ± 4	105 ± 2	91 ± 8
	FP	80 ± 1	84 ± 5	80 ± 2	101 ± 2	47 ± 3	-	65 ± 11	42 ± 1	87 ± 1	92 ± 3	95 ± 2
	CM	83 ± 4	84 ± 3	63 ± 10	110 ± 10	47 ± 2	-	77 ± 4	32 ± 3	75 ± 2	86 ± 6	89 ± 5
Acid whey	WM	99 ± 10	95 ± 1	102 ± 3	79 ± 3	79 ± 2	93 ± 4	41 ± 2	46 ± 0	140 ± 6	112 ± 5	60 ± 3
	WS	94 ± 5	107 ± 9	106 ± 7	59 ± 7	83 ± 3	-	31 ± 2	55 ± 3	137 ± 9	113 ± 8	30 ± 1
	FP	81 ± 2	82 ± 1	79 ± 6	96 ± 6	41 ± 2	-	71 ± 2	43 ± 2	96 ± 3	91 ± 2	30 ± 2
	CM	81 ± 1	81 ± 3	82 ± 12	101 ± 12	47 ± 2	-	97 ± 1	44 ± 1	87 ± 2	91 ± 5	66 ± 5

Table 2: Protein recovery rates (%) estimated using different extractants to isolate egg proteins out of egg material or out of egg material in matrix
(WM = without matrix; WS = wheat starch; FP = fat powder; CM = cake mix)

		1	2	3	4	5	6	7	8	9	10	11	12
Whole egg	WM	27 ± 2	26 ± 2	20 ± 1	23 ± 2	108 ± 3	74 ± 5	26 ± 1	22 ± 1	119 ± 11	112 ± 3	64 ± 2	87
	WS	22 ± 1	22 ± 2	22 ± 2	21 ± 1	108 ± 10	-	21 ± 2	27 ± 2	100 ± 4	117 ± 4	70 ± 0	84
	FP	20 ± 1	22 ± 1	18 ± 2	41 ± 2	75 ± 1	-	22 ± 2	30 ± 2	125 ± 0	134 ± 5	64 ± 6	97
	CM	29 ± 2	25 ± 1	21 ± 4	28 ± 3	78 ± 1	-	27 ± 3	30 ± 3	100 ± 2	124 ± 4	45 ± 2	112
Egg yolk	WM	37 ± 2	36 ± 1	38 ± 0	30 ± 2	86 ± 0	63 ± 4	79 ± 2	49 ± 2	129 ± 6	113 ± 5	16 ± 0	71
	WS	38 ± 1	38 ± 2	39 ± 4	30 ± 2	83 ± 4	-	62 ± 1	45 ± 1	111 ± 9	108 ± 2	20 ± 1	73
	FP	81 ± 6	70 ± 4	70 ± 11	75 ± 4	77 ± 2	-	86 ± 6	33 ± 2	151 ± 9	139 ± 4	94 ± 7	90
	CM	38 ± 2	38 ± 1	50 ± 3	45 ± 4	85 ± 5	-	107 ± 8	35 ± 3	113 ± 12	133 ± 6	51 ± 1	83
Egg white	WM	76 ± 2	76 ± 7	61 ± 11	53 ± 1	121 ± 3	116 ± 8	37 ± 2	20 ± 0	138 ± 16	123 ± 5	77 ± 2	86
	WS	76 ± 3	79 ± 1	71 ± 1	69 ± 1	120 ± 3	-	39 ± 1	21 ± 2	135 ± 9	132 ± 2	88 ± 2	95
	FP	15 ± 1	14 ± 0	19 ± 0	16 ± 0	90 ± 1	-	39 ± 2	32 ± 1	130 ± 10	145 ± 4	24 ± 2	111
	CM	16 ± 2	19 ± 2	22 ± 8	26 ± 4	78 ± 0	-	52 ± 6	44 ± 1	120 ± 4	141 ± 9	41 ± 2	102

Table 3: Protein recovery rates (%) using solution I and J, respectively, at different pH-values to isolate milk proteins from milk material and from milk material in certain matrices at different temperatures. (WM = without matrix; WS = wheat starch; CM = cake mix)

Dry milk material	Extractant	Matrix	pH	Recovery rates [%]		
				4 °C	RT	40 °C
Milk powder	I	WM	7.4	95 ± 6	103 ± 5	96 ± 4
			9.4	72 ± 7	82 ± 9	89 ± 7
			11.4	90 ± 4	91 ± 2	95 ± 3
		WS	7.4	95 ± 2	71 ± 4	85 ± 6
			9.4	63 ± 4	59 ± 2	68 ± 6
		CM	7.4	97 ± 2	89 ± 1	89 ± 3
			9.4	60 ± 3	62 ± 6	57 ± 5
	J	WM	8.5	73 ± 3	75 ± 5	74 ± 5
			10.5	83 ± 2	83 ± 3	78 ± 3
			12.5	99 ± 2	93 ± 4	90 ± 5
		WS	8.5	90 ± 1	96 ± 6	96 ± 5
			10.5	92 ± 4	91 ± 5	92 ± 1
		CM	8.5	98 ± 3	97 ± 5	99 ± 6
			10.5	112 ± 7	108 ± 2	115 ± 6
Sweet whey	I	WM	7.4	85 ± 2	87 ± 3	100 ± 3
			9.4	82 ± 1	90 ± 3	79 ± 6
			11.4	111 ± 4	107 ± 1	105 ± 2
		WS	7.4	80 ± 5	70 ± 4	80 ± 3
			9.4	76 ± 6	87 ± 5	81 ± 3
		CM	7.4	89 ± 3	83 ± 3	87 ± 1
			9.4	76 ± 3	70 ± 1	75 ± 6
	J	WM	8.5	67 ± 3	72 ± 1	77 ± 4
			10.5	88 ± 0	91 ± 1	89 ± 4
			12.5	129 ± 11	118 ± 11	110 ± 2
		WS	8.5	103 ± 5	102 ± 3	109 ± 7
			10.5	101 ± 1	100 ± 1	101 ± 1
		CM	8.5	113 ± 4	111 ± 1	114 ± 1
			10.5	118 ± 2	113 ± 2	111 ± 1
Acid whey	I	WM	7.4	81 ± 5	95 ± 2	96 ± 1
			9.4	91 ± 1	91 ± 10	89 ± 4
			11.4	109 ± 6	128 ± 12	109 ± 8
		WS	7.4	84 ± 3	87 ± 4	87 ± 3
			9.4	81 ± 2	49 ± 5	88 ± 7
		CM	7.4	80 ± 7	97 ± 4	95 ± 3
			9.4	87 ± 7	85 ± 4	86 ± 2
	J	WM	8.5	71 ± 2	75 ± 4	76 ± 1
			10.5	87 ± 2	98 ± 6	83 ± 2
			12.5	113 ± 2	113 ± 3	101 ± 4
		WS	8.5	100 ± 2	102 ± 2	101 ± 3
			10.5	96 ± 3	100 ± 5	101 ± 1
		CM	8.5	118 ± 12	115 ± 4	111 ± 3
			10.5	108 ± 2	119 ± 7	108 ± 11

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Table 4: Protein recovery rates (%) using solution I and J, respectively, at different pH-values to isolate egg proteins from egg material and from egg material in certain matrices at different temperatures. (WM = without matrix; WS = wheat starch; CM = cake mix)

Dry egg material	Extractant	Matrix	pH	Recovery rates [%]		
				4 °C	RT	40 °C
Whole egg	I	WM	7.4	94 ± 1	89 ± 7	99 ± 5
			9.4	102 ± 4	98 ± 8	101 ± 2
			11.4	110 ± 3	103 ± 4	94 ± 6
		WS	7.4	93 ± 4	93 ± 1	92 ± 4
			9.4	102 ± 3	101 ± 1	101 ± 8
		CM	7.4	100 ± 3	105 ± 9	105 ± 2
			9.4	105 ± 1	105 ± 2	106 ± 3
	J	WM	8.5	114 ± 3	120 ± 2	126 ± 4
			10.5	109 ± 2	117 ± 3	114 ± 2
			12.5	110 ± 3	111 ± 7	111 ± 6
		WS	8.5	111 ± 5	117 ± 7	114 ± 9
			10.5	102 ± 6	108 ± 4	113 ± 5
		CM	8.5	111 ± 7	113 ± 5	106 ± 1
			10.5	92 ± 4	91 ± 4	100 ± 2
Egg white	I	WM	7.4	114 ± 8	109 ± 9	117 ± 3
			9.4	116 ± 4	115 ± 3	127 ± 6
			11.4	126 ± 6	123 ± 7	118 ± 3
		WS	7.4	107 ± 6	115 ± 5	116 ± 5
			9.4	123 ± 7	123 ± 9	126 ± 1
		CM	7.4	114 ± 2	114 ± 4	121 ± 7
			9.4	118 ± 5	120 ± 4	127 ± 10
	J	WM	8.5	102 ± 4	122 ± 2	131 ± 2
			10.5	119 ± 7	116 ± 1	126 ± 6
			12.5	118 ± 6	124 ± 6	120 ± 7
		WS	8.5	124 ± 8	119 ± 8	127 ± 2
			10.5	126 ± 4	116 ± 5	119 ± 3
		CM	8.5	123 ± 11	122 ± 3	118 ± 5
			10.5	105 ± 6	109 ± 6	114 ± 4
Egg yolk	I	WM	7.4	121 ± 2	121 ± 2	132 ± 11
			9.4	125 ± 10	111 ± 6	103 ± 5
			11.4	116 ± 1	111 ± 7	112 ± 7
		WS	7.4	106 ± 1	101 ± 0	103 ± 7
			9.4	118 ± 7	109 ± 5	114 ± 8
		CM	7.4	105 ± 3	106 ± 3	105 ± 7
			9.4	115 ± 3	121 ± 7	113 ± 2
	J	WM	8.5	108 ± 1	110 ± 10	121 ± 8
			10.5	117 ± 2	113 ± 1	117 ± 6
			12.5	103 ± 6	109 ± 7	105 ± 3
		WS	8.5	115 ± 4	112 ± 3	114 ± 5
			10.5	111 ± 1	110 ± 2	111 ± 4
		CM	8.5	113 ± 4	113 ± 10	111 ± 5
			10.5	92 ± 6	87 ± 3	96 ± 6

Table 5: Protein recovery rates obtained by varied extraction durations at three temperatures using solution I (pH 7.4) and J (pH 8.5) to isolate milk proteins from skim milk (WM = without matrix; CM = cake mix).

Extractant	Matrix	Temp.	Extraction duration [h]					
			0	0.5	1	2	4	8
J (pH 8.5)	WM	4 °C	81 ± 1	82 ± 0	90 ± 1	85 ± 2	85 ± 6	82 ± 2
		RT		81 ± 2	97 ± 1	89 ± 3	80 ± 7	85 ± 4
		40 °C		82 ± 3	89 ± 3	78 ± 1	82 ± 3	84 ± 3
	CM	4 °C	80 ± 1	89 ± 8	98 ± 3	92 ± 7	99 ± 2	88 ± 5
		RT		80 ± 6	90 ± 9	98 ± 5	75 ± 7	86 ± 0
		40 °C		82 ± 4	88 ± 7	87 ± 1	96 ± 2	91 ± 7
I (pH 7.4)	WM	4 °C	96 ± 1	88 ± 1	101 ± 9	84 ± 2	86 ± 1	82 ± 0
		RT		94 ± 2	102 ± 4	86 ± 0	114 ± 9	86 ± 1
		40 °C		84 ± 7	112 ± 8	83 ± 1	87 ± 1	88 ± 8
	CM	4 °C	78 ± 4	90 ± 4	83 ± 1	80 ± 5	86 ± 2	83 ± 1
		RT		80 ± 0	90 ± 6	83 ± 4	80 ± 6	77 ± 7
		40 °C		83 ± 3	92 ± 5	76 ± 7	93 ± 9	78 ± 3

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Table 6: Protein recovery rates obtained by varied extraction durations at three temperatures using solution I and J to isolate egg proteins from whole egg powder (WM = without matrix; CM = cake mix).

Extractant	Matrix	Temp.	Extraction duration [h]					
			0	0.5	1	2	4	8
J (pH 8.5)	WM	4 °C	107 ± 4	106 ± 5	105 ± 1	104 ± 4	106 ± 3	104 ± 4
		RT		110 ± 3	108 ± 3	104 ± 2	104 ± 1	109 ± 2
		40 °C		110 ± 0	109 ± 2	109 ± 4	106 ± 3	107 ± 1
	CM	4 °C	115 ± 6	116 ± 6	105 ± 3	112 ± 1	105 ± 4	113 ± 8
		RT		119 ± 12	117 ± 5	112 ± 4	111 ± 3	112 ± 6
		40 °C		116 ± 0	122 ± 3	120 ± 2	123 ± 0	111 ± 6
I (pH 7.4)	WM	4 °C	94 ± 6	96 ± 2	99 ± 2	99 ± 5	104 ± 2	104 ± 3
		RT		96 ± 4	93 ± 3	98 ± 2	94 ± 3	96 ± 6
		40 °C		99 ± 3	103 ± 3	95 ± 2	101 ± 0	101 ± 3
	CM	4 °C	91 ± 2	92 ± 1	93 ± 1	92 ± 1	89 ± 6	91 ± 0
		RT		90 ± 0	99 ± 1	93 ± 2	94 ± 4	93 ± 5
		40 °C		112 ± 3	112 ± 6	117 ± 3	117 ± 2	108 ± 0

Fig. 1: LDS - PAGE and silver staining patterns of proteins extracted from egg powder (left) and milk powder (right), respectively, using different extractants. MW: molecular weight marker; CON: Conalbumin (76 kDa); OVA: Ovalbumin (44.5 kDa); OVO: Ovomucoid (28 kDa); LYS: Lysozyme (14.3 kDa); BSA: Bovine serum albumin (66.3 kDa); CAS: Caseins (20.6 -25.2 kDa); LG: β -lactoglobulin (18.3 kDa); LA: α -lactalbumin (14.2 kDa).

Fig. 2: LDS - PAGE and silver staining patterns of proteins extracted from egg powder using solution J (left) and I (right), respectively, adjusted to different pH-values and used for extraction at 4 °C, room temperature (RT) and 40 °C. MW: molecular weight marker; CON: Conalbumin (76 kDa); OVA: Ovalbumin (44.5 kDa); OVO: Ovomucoid (28 kDa); LYS: Lysozyme (14.3 kDa).

Fig. 3: Left: LDS - PAGE and Immunoblotting of ovalbumin extracted from egg powder using different extractants and detection via anti-ovalbumin antibody. Right: LDS - PAGE and Immunoblotting of casein extracted from milk powder using different extractants and detection via anti-casein antibody. MW: molecular weight marker; CON: Conalbumin (76 kDa); OVA: Ovalbumin (44.5 kDa); OVO: Ovomucoid (28 kDa); LYS: Lysozyme (14.3 kDa); BSA: Bovine serum albumin (66.3 kDa); CAS: Casein (20.6 -25.2 kDa).

Fig. 4: Left: LDS - PAGE and Immunoblotting of ovalbumin extracted from egg powder out of dry cake mix as matrix using different extractants and detection via anti-ovalbumin antibody. Right: LDS - PAGE and Immunoblotting of casein extracted from milk powder out of dry cake mix as matrix using different extractants and detection via anti-casein antibody. MW: molecular weight marker; CON: Conalbumin (76 kDa); OVA: Ovalbumin (44.5 kDa); OVO: Ovomucoid (28 kDa); LYS: Lysozyme (14.3 kDa); BSA: Bovine serum albumin (66.3 kDa); CAS: Casein (20.6 -25.3 kDa).

Figure 1
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Figure 2
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