

The Effect of the Food Matrix on the In Vitro Bio-Accessibility and IgE Reactivity of Peanut Allergens

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Scope: Factors such as food processing, the food matrix, and antacid medication may affect the bio-accessibility of proteins in the gastrointestinal tract and hence their allergenic activity. However, at present they are poorly understood.

Methods and Results: Roasted peanut flour was incorporated into either a chocolate dessert or cookie matrix and bio-accessibility were assessed using an in vitro digestion system comprising a model chew and simulated gastric and duodenal digestion. Protein digestion was monitored by SDS-PAGE and immunoreactivity analyzed by immunoblotting and immunoassay. IgE reactivity was assessed by immunoassay using serum panels from peanut-allergic subjects. Roasted peanut flour proteins proved highly digestible following gastro-duodenal digestion even when incurred into a food matrix, with only low molecular weight polypeptides of $M_r < 8$ kDa remaining. When gastric digestion was performed at pH 6.5 (simulating the effect of antacid medication), peanut proteins are not digested; subsequent duodenal digestion is also limited. IgE reactivity of the major peanut allergens Ara h 1, Ara h 2, and Ara h 6, although reduced, was retained after oral-gastro-duodenal digestion irrespective of digestion conditions employed. **Conclusion:** Peanut allergen bio-accessibility is unaffected by the dessert or cookie matrices whilst high intra-gastric pH conditions render allergens more resistant to digestion.

1. Introduction

Peanut is a frequent cause of IgE-mediated reactions across the world, affecting around 2% of school-age children in the UK^[1] and four-year-old children in Australia,^[2] although the estimated prevalence of challenge-proven peanut allergy across Europe is only 0.5%.^[3] Peanut allergy can be severe as indicated in a recent analysis of fatal allergic reaction in the UK, which showed, that 16–20% of incidents where the incriminated food was recorded were due to peanut.^[4] Peanut was also the most frequently reported food causing anaphylaxis in the European Network of severe allergic reactions.^[5]

More than ten different proteins have been identified as allergens in peanut.^[6,7] These include the 7S and 11S seed storage globulins belonging to the cupin superfamily (known as Ara h 1 and Ara h 3 respectively) together with members of the prolamin superfamily including 2S albumins (known as Ara h 2, 6, and 7) and lipid transfer protein (Ara h 9). Component-resolved diagnosis has shown that sensitization to these

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prolamin and cupin superfamily allergens is associated with clinical allergy to peanut.^[8–10] Whilst Ara h 1 is rapidly degraded by the action of pepsin, the 2S albumin allergens are highly resistant to pepsin, although both types of allergen retain their IgE-reactivity.^[11,12] and despite being degraded Ara h 1 retains its sensitizing capacity.^[13] The 2S albumin allergens are also resistant to duodenal proteases such as trypsin.^[14] Investigating the effects of thermal processing on allergens is complex and conflicting results have been obtained when studying the effect of heating on allergenicity of purified peanut proteins^[15–17] compared to whole peanut.^[18–21] Few studies have investigated the effects of incurring allergenic food ingredients into processed food matrices. However, changes in the recipes of foods used for diagnosis of allergy in oral food challenges to improve blinding by increasing the fat content appear to enhance the allergenic activity of peanut and hazelnut in terms of symptom severity, and (possibly) threshold dose.^[22,23] Such observations have given rise to the hypothesis that the food matrix may affect allergenicity by altering the progressive release of allergens from the food matrix and their susceptibility to digestion. This in turn affects their accessibility to the gut epithelium—a property often termed “bio-accessibility.”^[24] Bio-accessibility is considered to be a measure of the amount of a substance that is released from a food matrix in the gastrointestinal tract and becomes available for intestinal absorption.^[25] The bio-accessibility of allergens could affect the way in which they either sensitize or elicit an allergic reaction and may be modified in individuals with an altered digestive capacity such as infants^[26] and those taking antacid medication. In vitro models of digestion have been developed which allow the effects of the food processing and the food matrix on allergen bio-accessibility to be assessed.^[27] Models simulating gastrointestinal digestion have revealed complex matrix effects, which can reduce the digestibility of gluten proteins^[28] and caseins,^[29] whilst adsorption to oil droplets and lipid vesicles can either enhance or reduce protein digestibility.^[30,31]

As part of the EU-funded iFAAM project, the factors governing the bio-accessibility of peanut allergens in different food matrices was investigated using an in vitro digestion model^[28] and the impact on IgE-binding capacity determined using serum panels from peanut allergic individuals. A well characterized roasted peanut flour was incurred in either a water-continuous chocolate dessert matrix used previously for double blinded placebo controlled oral food challenges^[32] or a baked cookie matrix chosen to represent a low water, high carbohydrate, and fat matrix, where the peanut flour was incurred in a gluten network and subjected to a further thermal treatment.

2. Results and Discussion

2.1. Effect of Different Food Matrices and Intra-Gastric pH on the In Vitro Digestibility of Peanut Proteins

In order to take into account the fact that the body adjusts the volume of gastric secretions depending on the macro-nutrient composition of food consumed^[27] the in vitro oral-gastric digestion conditions were adjusted for each matrix based on their proximate composition (Table S4 and Figure S1, Supporting Information). This, together with the fact the peanut protein com-

prised between 2–10% (w/w) of the incurred food matrices, made direct comparison of digestion profiles complex (Figure S1 and Table S7, Supporting Information). Digestions were undertaken using gastric pH values of 2.5 and 6.5 to understand how the use of antacid medication might affect allergen bio-accessibility and allergenicity.^[33] The soluble fraction of the digests was the main focus of this study since it represents the major bio-accessible fraction of the allergens (or derived fragments). Only limited characterization of the more complex and heterogeneous food particulates was possible with the methods available. In order to conserve human serum samples, samples were selected for IgE immunoblotting based on analysis performed using animal antibodies specific to purified peanut allergens.

Initially the digestibility of roasted peanut flour proteins was investigated (Figure 1; Figure S2, Supporting Information). As previously described^[34,35] the peanut flour profile is dominated by the $M_r \approx 38$ and ≈ 23 kDa polypeptides corresponding respectively to the acidic and basic polypeptides of Ara h 3. The $M_r \approx 62$ kD and ≈ 14 kDa polypeptides, corresponding respectively to Ara h 1 and Ara h 2, were only faintly visible from the protein stained gel of the roasted peanut. However, together with Ara h 6, they were clearly detected by immunoblotting, although with evidence of aggregation (Figure S3, Supporting Information). Simulated salivary fluid solubilized a proportion of the protein (Figure 1A lane C) which, unlike the undigested peanut flour, showed little evidence of higher $M_r > 50000$ protein aggregates (Figure 1B lane C), the profile of which is altered following addition of simulated gastric fluid (SGF) (Figure 1A lane GU). Low pH gastric digestion resulted in loss of the peanut flour proteins from the insoluble fraction after only 2 min. This was accompanied by the development of a stable polypeptide profile in the soluble fraction comprising faintly staining undigested polypeptides together with intensely staining material of $M_r < 10$ kDa, much of it poorly resolved (Figure 1B lane 0.3–11 min). These changes were accompanied by a loss of Ara h 1 and Ara h 3 immunoreactivity in the soluble fractions, although the immunoreactivity of Ara h 2 and 6 was unaltered throughout digestion (Figure S3, A–C, Supporting Information). The 11 min pH 2.5 gastric digest was selected for duodenal digestion as it represented a stable end-point of the digestion relative to the full 120 min digest. Duodenal digestion resulted in rapid degradation of any remaining soluble material, although some insoluble material did remain (Figure 1E,F; Figure S3, Supporting Information). Gastric digestion at pH 6.5 resulted in the peanut protein remaining in the insoluble fraction and not being degraded (Figure 1C,D). The peanut allergens (Ara h 1, the acidic subunit of Ara h 3, Ara h 2, and 6) also retained their immunoreactivity on immunoblotting analysis of the soluble fraction with no change in signal intensity over time (Figure S2D–F, Supporting Information). Subsequent duodenal digestion of the 11 min pH 6.5 gastric digest showed incomplete digestion with significant peanut protein remaining in the insoluble phase, which appeared to be gradually, but only partially, digested over the time course of duodenal digestion (Figure 1G,H). No immunoreactive Ara h 1 was detected by immunoblotting of the soluble fraction (data not shown) and immunoblotting analysis only showed evidence of the acidic subunit of Ara h 3 in the undigested duodenal control sample and the 0.3 min time point (Figure S4, Supporting Information).

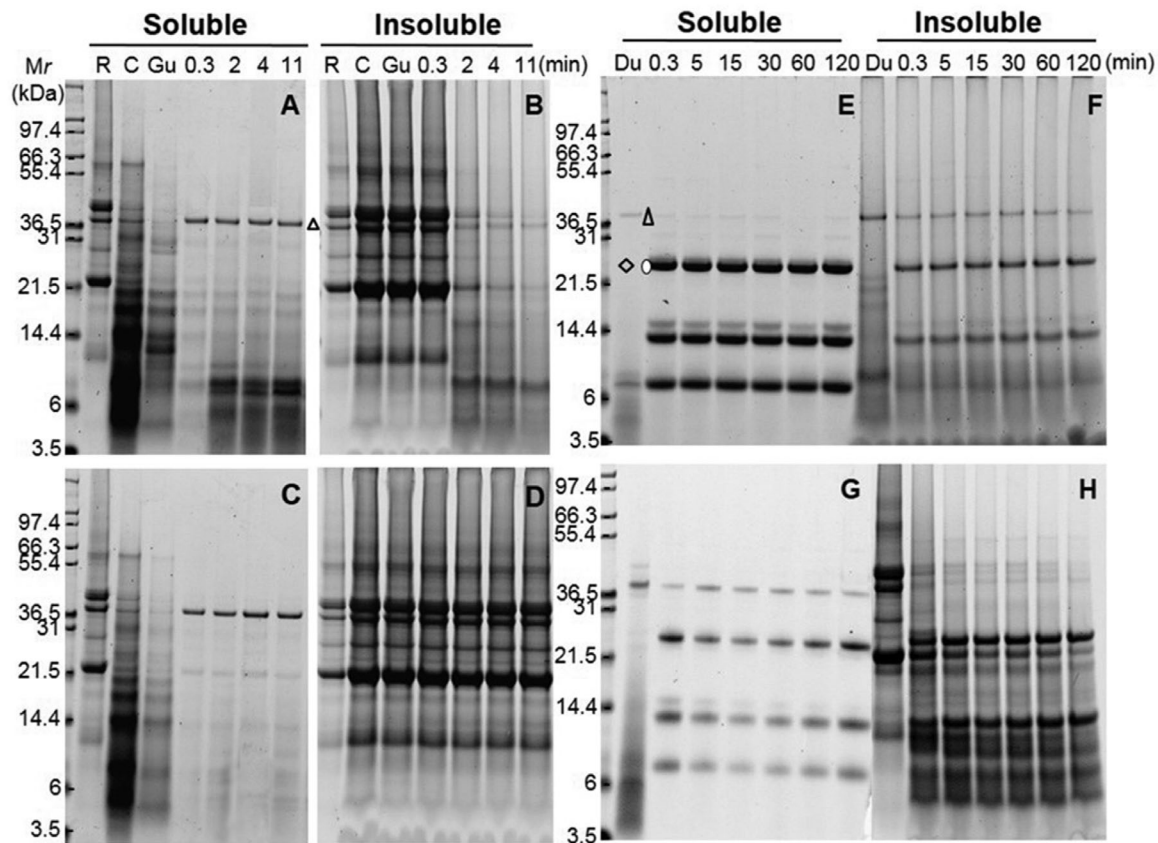


Figure 1. A–D) SDS-PAGE analysis of simulated oral-gastro and E–H) oral-gastro-duodenal digestion of peanut flour. Gastric digests were performed at pH 2.5 (A, B) and pH 6.5 (C, D) and duodenal digestion performed on the 11 min gastric digestions at pH 2.5 (E, F) and pH 6.5 (G, H). Soluble (A, C, E, G) and insoluble fractions (B, D, F, H) were analyzed by SDS-PAGE. R—un-digested chaotropic reducing buffer extract of peanut flour; C—chew sample; Gu—no enzyme gastric control; Du—no enzyme duodenal control; Panels A–D lanes 1 to 11—0.3, 2, 4, 11, 22, 33, 44, 55, 66, 77, and 120 min; panels E–H lanes 1 to 6—0.3, 5, 15, 30, 60, and 120 min. Δ: pepsin; ○: trypsin; ◇: chymotrypsin. Peanut flour soluble digests were diluted 1:10 (v:v) prior to analysis and 10 μ L loaded per well and consequently α -amylase was not evident.

Subsequently, the effect of incurring the peanut flour into two food matrices on allergen digestibility was investigated. The first was a chocolate dessert matrix containing cocoa powder and comprising a cold swelling starch gel, which has been used for oral food challenges in the EuroPrevall project.^[22,31] This matrix itself contains little protein apart from the residual, highly modified cocoa proteins, as is indicated by SDS-PAGE analysis of digests of the matrix alone without peanut (Figure S5, Supporting Information). SDS-PAGE analysis of the simulated oral-gastro digestion at pH 2.5 of the peanut-containing matrix showed that the peanut proteins largely retained their highly digestible nature although were faintly staining due to the level of inclusion in the dessert matrix (Figure 2). A range of polypeptides was solubilized in the chew sample, which was slightly modified in the low pH no-enzyme gastric control (GU) sample (Figure 2A lanes C and GU). The low Mr < 6 kDa digestion products in the soluble fraction of the gastric digestion time course were not as well resolved as for the peanut flour alone (Figure 1A), probably because of other components in the dessert matrix (Figure 2A). The peanut proteins in the insoluble phase also appeared to persist for a slightly longer time in the dessert matrix compared to the peanut flour alone (4 min rather than 0.3 min; Figure 2B

lanes 1–3). Immunoblotting analysis of the soluble fractions of the dessert digest showed that Ara h 1 was not present in an immuno-reactive form in the soluble phase of the chew although it was solubilized in the no-enzyme GU control. However, Ara h 1 and Ara h 3 immunoreactivity was completely lost after 11 min gastric digestion (Figure S7 A,B, Supporting Information). In contrast, Ara h 2 and 6 remained largely intact and highly immune-reactive for 120 min of gastric digestion (Figure S7 C, Supporting Information). As with the peanut flour gastric digest, the 11 min time point was selected for duodenal digestion since it reflected a stable end-point of the gastric digestion. There was no evidence of peanut protein digestion products in the soluble fraction of a duodenal digest of the 11 min pH 2.5 gastric time point with only a persistent, faintly staining poorly resolved low Mr material being observed in the insoluble fraction (Figure 2F, lanes 1–6). Ara h 3 retained its immunoreactivity only in the 0.3 min duodenal digest time point although it was accompanied by weakly staining Mr $\approx$ 24 and $\approx$ 10 kDa fragments, which persisted for 30 min (Figure S7, Supporting Information). When in vitro gastric digestion of the dessert matrix was undertaken at pH 6.5 no digestion of peanut proteins was observed (Figures 2C,D). Ara h 1 was only weakly immunoreactive in

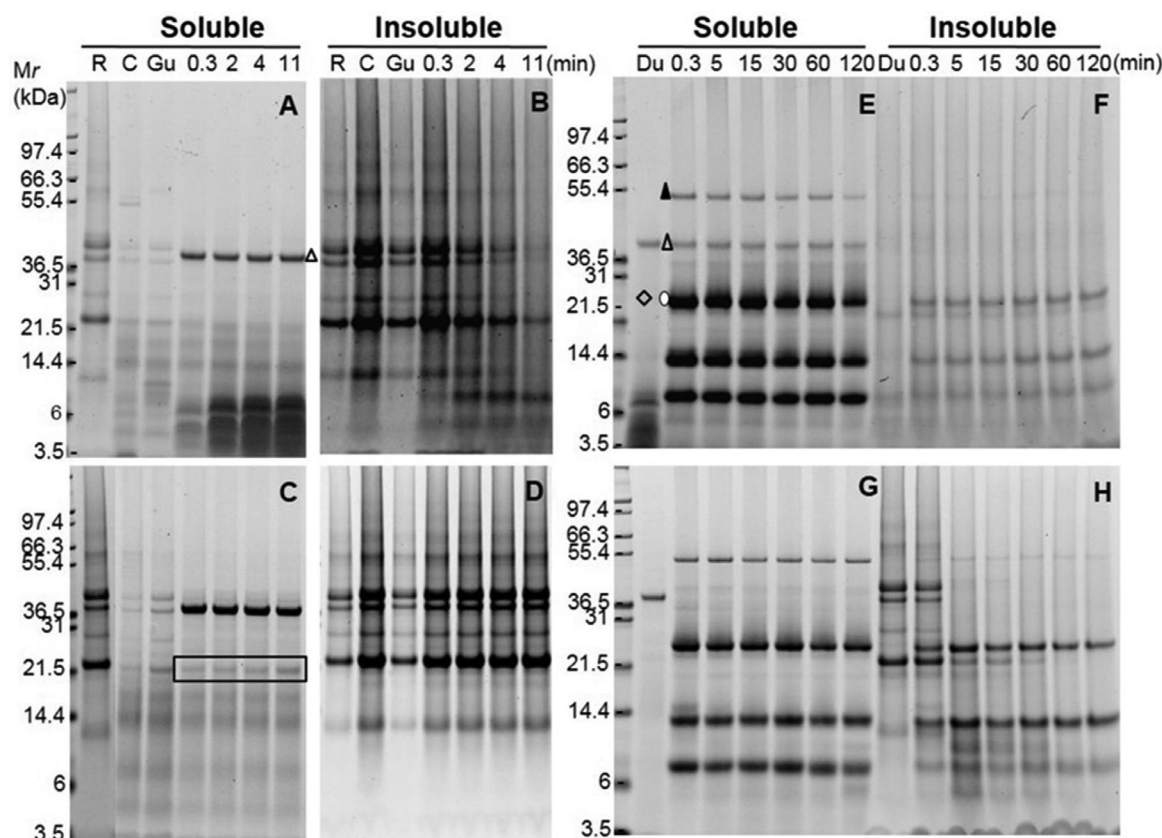


Figure 2. A–D) SDS-PAGE analysis of simulated oral-gastric and E–H) oral-gastro-duodenal digestion of dessert matrix containing peanut flour. Figure annotation is as described for Figure 1; ▲: α -amylase.

the soluble fraction, whereas more immunoreactive intact Ara h 3, Ara h 2, and 6 passed into the soluble fraction (Figure S7 D–F, Supporting Information). Analysis of the 11 min pH 6.5 gastric digest subjected to duodenal digestion also showed no evidence of peanut flour digestion products in the soluble phase (Figure 2G), although transient polypeptides of Mr $\approx$ 24 and 10 kDa were observed in the insoluble fraction which persisted for 30 min of duodenal digestion (Figure 2H). These polypeptides were stained less intensely than those observed in the peanut flour, although this in part reflects the fact the dessert contains only $\approx$ 15% (w/w) of the peanut protein present in the flour digests (Figure S8, Supporting Information).

Cookie matrix analysis by SDS-PAGE was more complicated due to the presence of gluten polypeptides (Figure S9, Supporting Information) and as a result the peanut proteins were less evident, particularly the acidic subunit of Ara h 3 (Figure 3A–D; Figure S10, Supporting Information). Significant amounts of undigested protein were observed in the insoluble fraction of the cookie digest performed at pH 2.5 even after 120 min of digestion, a pattern reminiscent of that previously observed for bread digests.^[28] Immunoblotting analysis of the soluble fraction of the digests indicated that whilst Ara h 1 and Ara h 3 immunoreactivity was lost, that of Ara h 2 and 6 was retained (Figure S11 A–C, Supporting Information). Duodenal digestion of the pH 2.5 11 min gastric time point was then undertaken, since it was again representative of the stable end-point of gastric digestion

as indicated by SDS-PAGE analysis (Figure 3). No immunoreactivity towards Ara h 1 was observed (data not shown), although two faintly staining Ara h 3 reactive polypeptides of Mr $\approx$ 23 and 12 kDa persisted for 60 min (Figure S12, Supporting Information). However, these fragments did not appear to correspond to any of the strongly staining polypeptides evident in the protein stained gel (Figure 3A). Again when gastric digestion was performed at pH 6.5 proteins were undigested in both the soluble and insoluble fractions (Figure 3C,D) and immunoreactivity with all three antibody preparations was retained (Figure S8 D–F, Supporting Information). Following duodenal digestion of the pH 6.5 11 min gastric digest, there was evidence of low molecular weight (Mr < 10 kDa) digestion products in the soluble fraction (Figure 3G). Higher molecular weight digestion products, including a Mr $\approx$ 12 kDa polypeptide, were also evident in the insoluble phase (Figure 3H) which may correspond to one of the Ara h 3 fragments identified by immunoblotting (Figure S9, Supporting Information).

2.2. Effect of In Vitro Digestion on the IgE-Reactivity of Peanut in Different Food Matrices

The effect of the different matrices on the stability of IgE binding of peanut allergens to in vitro digestion was then assessed using selected time points of digestion and serum from peanut

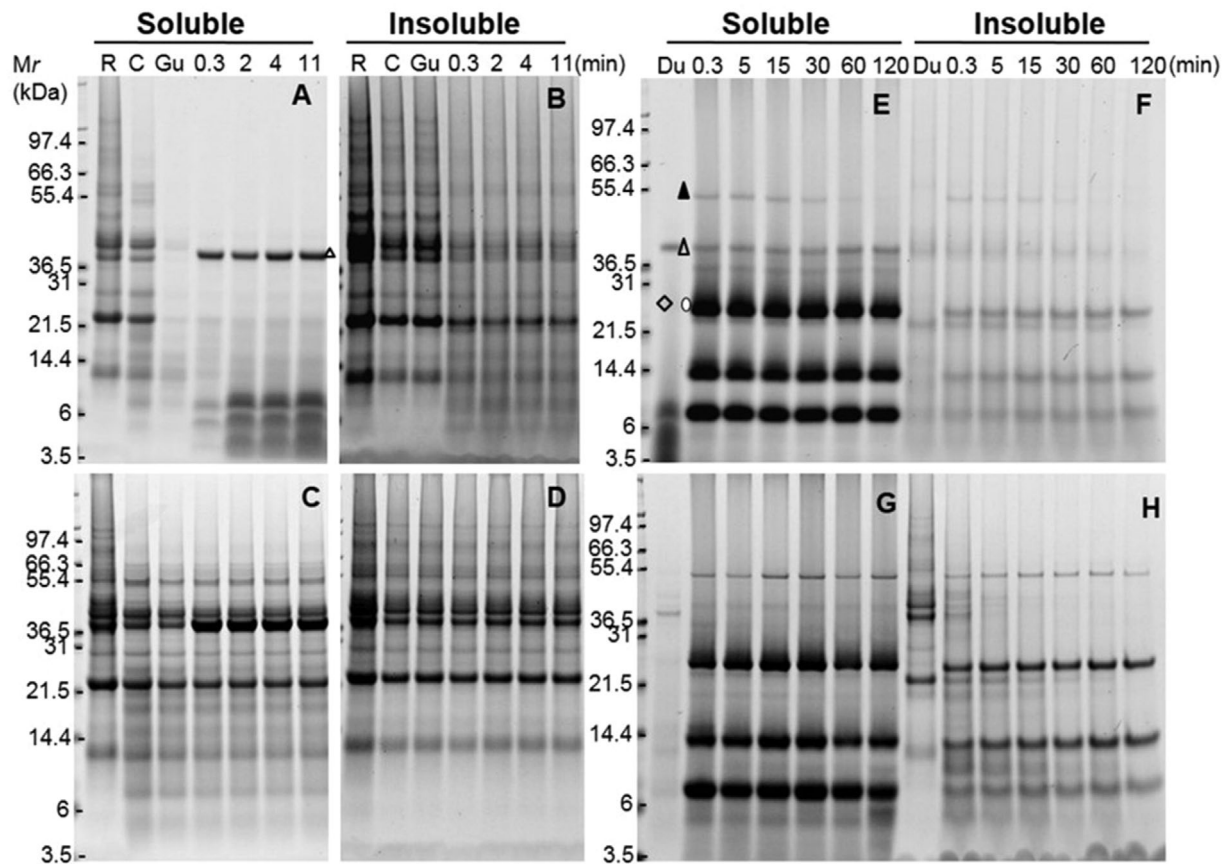


Figure 3. A–D) SDS-PAGE analysis of simulated oral-gastric and E–H) oral-gastro-duodenal digestion of cookie matrix containing peanut. Figure annotation is as described for Figure 1; ▲: α -amylase.

allergic subjects (Tables S5 and S6, Supporting Information). Immunoblotting analysis of the peanut flour extract (Figure 4, lane 1) revealed a heterogeneous pattern with most patient's serum IgE reacting to high molecular weight and aggregated proteins together with polypeptides corresponding to the 2S albumin allergens Ara h 2 and 6. IgE-binding to polypeptides corresponding to Ara h 2 and 6 was weak for patients 13, 11, and 12, and was absent for patient 15. This may reflect the fact the samples were reduced to ensure proteins were solubilized effectively from the food matrices,^[28] a factor known to affect IgE binding.^[36] In order to facilitate comparisons the peanut flour digest was diluted 10-fold to give the equivalent of 8.6 mg peanut protein in the starting material per mL of digest compared to 13 and 24 mg mL⁻¹, respectively for the dessert and cookie (Table S7, Supporting Information). Even taking this into consideration the IgE immunoblotting suggests that the IgE reactivity of Ara h 2 and 6 in the soluble fraction of digests was unaffected by the different matrices (Figure 4C, lanes 2–4). No IgE-reactivity was detected in the insoluble phase of the dessert (Figure 4C, lane 6) although IgE-binding to aggregated protein and Ara h 2 and 6 polypeptides was evident in the insoluble phase of the peanut flour and cookie for some patients (Figure 4C lanes 5, 7). Interestingly patients 13, 11, 12, and 15, whilst not reacting with Ara h 2/6 polypeptides in the peanut flour extract or the insoluble fractions of digests, did react to the proteins in the soluble fractions of digests. The pro-

cess of sonicating the peanut flour and insoluble fractions of the digests in urea-thiourea with reducing agent in order to solubilize protein may have reduced the IgE binding capacity of the Ara h 2 and 6 by disrupting conformational IgE-epitopes.^[36]

Subsequently the whole peanut-, Ara h 1-, Ara h 2-, and Ara h 6-related IgE binding capacities of the soluble fractions of the digests were assessed using competitive IgE-capture immunoassays employing plasma samples from four peanut allergic individuals (Table S6, Supporting Information). Digests were diluted to provide samples with equivalent amounts of peanut protein in the initial chew sample to develop inhibition curves, an example of which is given in Figure S13, Supporting Information. These were then used to derive concentrations able to inhibit binding to 50% (IC₅₀) values as a measure of IgE-reactivity. IC₅₀ values obtained using plasma samples from different patients were generally comparable as is illustrated for the peanut flour digests in Figure S14, Supporting Information. The median (Figure 5) and mean (Figure S14, Supporting Information) IC₅₀ values obtained for the different digests showed that the IgE binding capacity for whole peanut was not significantly modified during the gastric digestion for any samples (Figure 5A) even after 120 min digestion (data not shown). In contrast, the peanut-related IgE immunoreactivity of the soluble fractions progressively decreased during duodenal digestion, notably for the cookies and dessert matrices, as indicated by the 4-fold increase

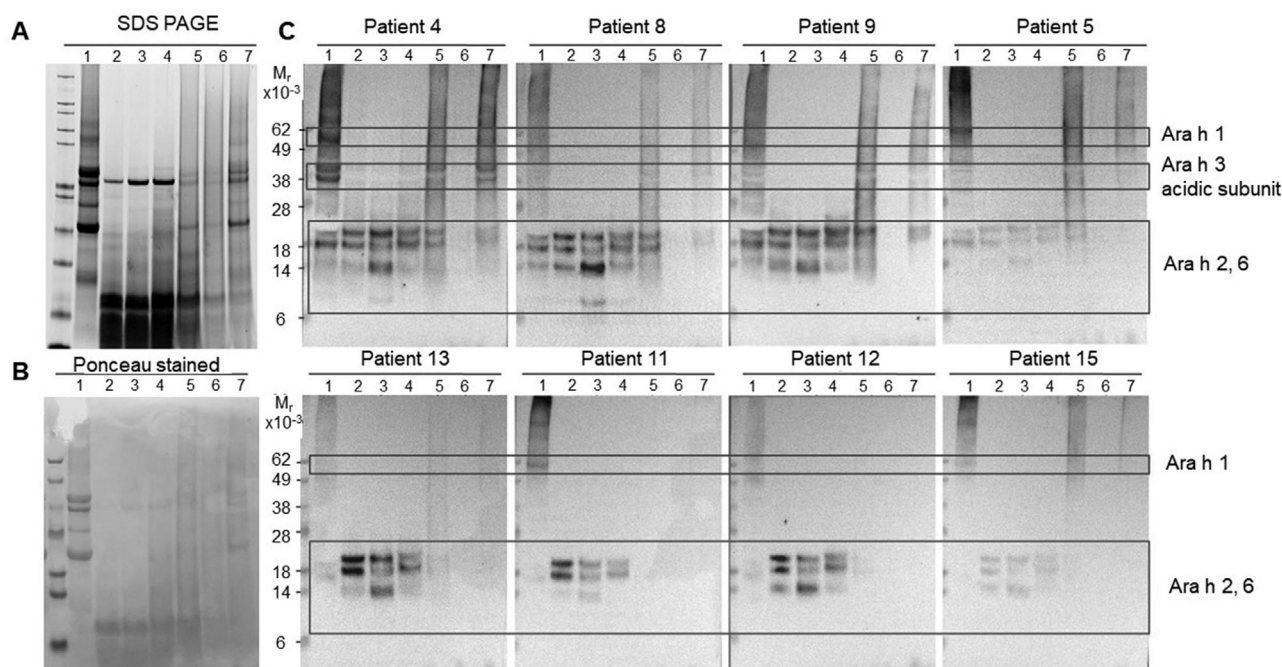


Figure 4. IgE immunoreactivity of digested peanut flour and peanut-containing matrices using plasma from peanut-allergic patients. Panel A: Coomassie blue stained gel; Panel B: Ponceau S stained blot; Panel C: Immunoblotting using plasma from peanut-allergic patients 4, 8, 9, 5, 13, 11, 12, and 15. Lane 1: un-digested chaotropic reducing buffer extract of peanut flour; 10 μ L per well was loaded of either soluble (lanes 2–4) or insoluble (lanes 5–7) fractions of 11 min gastric digestion performed at pH 2.5 of peanut flour (lanes 2,5; diluted 1:10 (v:v), peanut containing dessert (lanes 3, 6) and peanut-containing cookie (lanes 4,7).

in IC50 values for each of the patient plasma samples analyzed. However, IgE-immunoreactivity was not abolished and remained significant in the digests. Ara h 1-related IgE reactivity appeared lower in the chew samples of the dessert and cookie compared to the flour (Figure 5B). It then increased during the first minutes of gastric digestion, and was statistically significantly increased for the incurred gel dessert matrix and then remained stable for 120 min of gastric digestion. These data suggest immunoreactive Ara h 1 and/or fragments were progressively released from the matrix into the soluble fraction during gastric digestion. Ara h 1-related IgE reactivity then decreased following duodenal digestion, whatever the matrix, reaching initial values observed in chew material. Ara h 2 and Ara h 6-related IgE binding capacities were unaffected by either the matrix or gastric digestion (Figure 5C,D), even after 120 min of gastric digestion (data not shown). Subsequent duodenal digestion resulted in a limited but significant reduction in the IgE immunoreactivity of soluble Ara h 2 and Ara h 6. Gastric digestion at pH 6.5 had no effect on peanut IgE-reactivity (Supporting Information Figure S15). However, apart from Ara h 6, duodenal digestion led to a more extensive decrease in the IgE binding capacity than observed when gastric digestion was performed at pH 2.1 (Figure S15 D, Supporting Information).

3. Discussion

The results presented in this report indicate that the food matrix, regardless of either fat and water content or processing (baking), did not greatly affect the bio-accessibility of roasted peanut al-

lergens although there were some subtle differences in digestive behavior of the matrices and specific allergens. It appears that the un-aggregated allergens, and especially Ara h 2 and 6, were solubilized from the peanut flour and the matrices in a similar fashion by simulated salivary fluid. Consequently, all three forms of peanut would be expected to have the capacity to elicit reactions in the oral mucosa, an important warning sign of a potential allergic reaction for patients as has been identified by others.^[37] The bio-accessibility of Ara h 1 tended to increase during gastric digestion, as although largely degraded to polypeptides of $M_r < 10$ kDa, these digestion products retained their IgE-binding capacity, as has been observed for the purified protein.^[12] Following gastro-duodenal digestion the IgE reactivity of Ara h 1 was reduced more extensively, especially when incurred in the matrices. In contrast, the prolamin superfamily allergens Ara h 2 and Ara h 6 were highly resistant to digestion, again as has been observed previously for the purified allergens.^[21,35] Their allergenic activity was reduced to only a limited extent by gastroduodenal digestion and they therefore proved to be the most bio-accessible peanut allergens retaining their IgE-binding capacity in an almost unaltered form irrespective of the food matrix. It is likely that both intact protein and large, IgE-reactive fragments are responsible for this residual IgE binding capacity as has been described by others.^[38–40] For example, the IgE-reactive Mr14kDa polypeptide observed in the digests in this study (Figure 4) may correspond to a $M_r \approx 20$ kDa polypeptide observed by Di Stasio and coworkers which was identified by in-gel trypsin digestion and mass spectrometry as originating from Ara h 3.^[41]

These in vitro study data suggest that the matrices used in this study are unlikely to affect the clinical reactivity to peanut

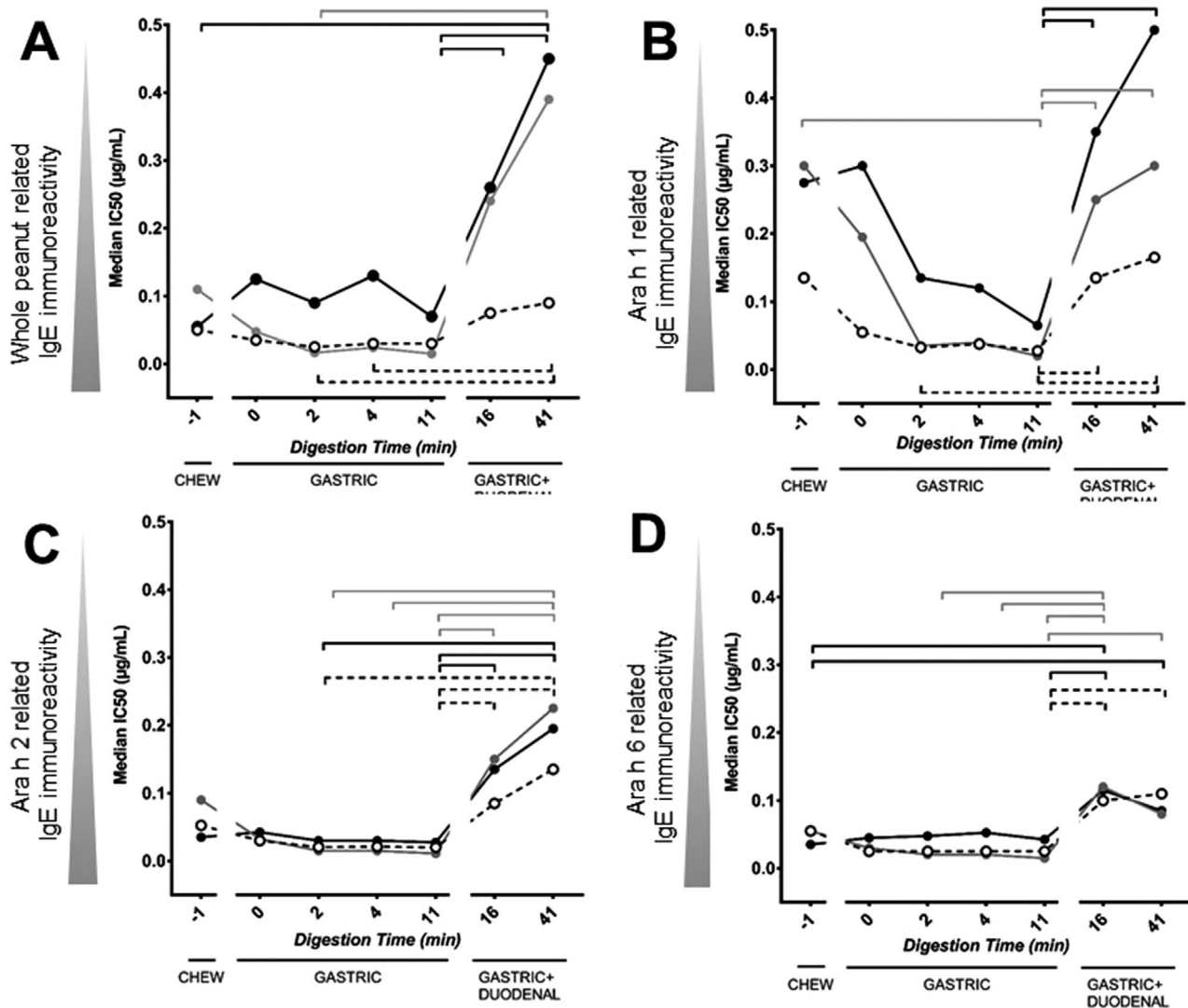


Figure 5. Comparison of the IgE binding capacity of oral-gastro-duodenal digesta from the peanut flour, peanut cookie and peanut dessert for gastric digestion performed at pH 2.5. Digests were diluted to give equivalent peanut protein concentrations and are indicated as follows: peanut flour: ---○---; peanut containing dessert matrix: —●—; peanut cookie: —●—. The human plasma IgE binding capacity is expressed as the median IC₅₀ value obtained for each digest from the different matrices to whole peanut protein extract (panel A), Ara h 1 (panel B), Ara h 2 (panel C), and Ara h 6 (panel D) using plasma for four peanut allergic patients. IC₅₀ values correspond to the protein concentration of a digestion sample, which inhibits the IgE binding to corresponding labelled allergens by 50%; an increase in IC₅₀ value therefore corresponds to a decrease in IgE-binding capacity. Bars indicate statistically significant differences (Friedman test, $p < 0.1$) between two different digestion time points within one matrix (peanut flour: ---○---; peanut containing dessert matrix: —●—; peanut cookie: —●—). No inhibition was observed using selected digestion time-points for matrices without peanut (data not shown).

and that the highly stable allergens, Ara h 2 and 6 make a major contribution to this allergenic activity. These allergens have also been shown to be highly resistant to in vitro oral-gastro-duodenal digestion from raw powdered peanut, although the roasted peanut flour used in this study appears to be better digested than raw peanut powder.^[38,39] It is likely that the high temperatures used in the roasting process may have enhanced peanut protein digestibility. The cookie was used as an exemplar baked matrix but it proved more digestible than a previously investigated bread matrix.^[27] It may therefore be that baked matrices, such as bread and cakes, will have a different effect on the clinical reactivity to food allergens.

As expected gastric digestion at high pH, mimicking the effect of taking antacids, results in no digestion by pepsin with little of the allergen becoming solubilized. This is reflected in the higher IgE binding capacity of these digesta. Instead, digestibility relied solely on pancreatic enzymes, which reduced the IgE binding capacity of the soluble fraction of the digests whichever matrix was analyzed. Duodenal digestion had a greater impact on reducing the IgE-binding capacity of the soluble fractions when the gastric digestion was performed at pH6.5. It seems likely this is because a greater proportion of the peanut allergens remained in the insoluble fraction as adjudged by SDS-PAGE of these digests. The significant changes in digestibility observed using a gastric pH

of 6.5 suggest that antacid medication, such as proton pump inhibitors, will have more impact on allergen bio-accessibility and digestibility than the food matrix. However, since the antacid only affects the delivery of intact allergen into the duodenum it might be expected to alter only the severity of gastrointestinal or systemic reactions rather than those resulting from exposure of, for example, the oral mucosa and esophageal epithelium. Further clinical studies on the effect of food matrices and proton pump inhibitors on reactivity to peanut in allergic individuals will be required to confirm the relevance of the observations made in this in vitro study.

4. Experimental Section

Materials: Lightly roasted mechanically-defatted peanut flour manufactured by the Golden Peanut Company (Alfaretta, USA) was sourced from Byrd Mill, USA. Other food ingredients used for food matrix preparation and skimmed milk powder were obtained from local retail outlets. Digestive enzymes were obtained as summarized in Table S1, Supporting Information. Sodium taurocholate, sodium glycol-deoxycholate, cholesterol, phenylmethylsulfonyl fluoride (PMSF) were from Sigma-Aldrich (Dorset, UK). NuPAGE Bis-Tris (4–12%) gels, NuPAGE lithium dodecylsulphate (LDS) buffer (4X, pH 8.4), 2-(N-morpholino) ethanesulfonic acid running buffer, SimplyBlue safe stain, Mark 12, and SeeBlue prestained markers were from Invitrogen (Shropshire, UK). Nitrocellulose membrane (0.2 µm pore size) was from Bio-Rad Laboratories (Hertfordshire, UK). Mouse anti-human IgE antibody labeled with horseradish peroxidase (HRP) was from Southern Biotech, Cambridge Bioscience Ltd (Cambridge, UK). Goat-anti rabbit IgG antibodies labeled with HRP, and SuperSignal West Dura Extended Duration Substrate were from ThermoFisher Scientific (Hertfordshire, UK). Polyclonal rabbit antibodies to Ara h 1, Ara h 3, and Ara h 2 were obtained as previously described.^[32] All other chemical reagents were of analytical grade unless otherwise stated.

Food Matrices: The protein content of the roasted peanut flour was determined to be 47.6% ($n = 3$) by the Kjeldahl method using the nitrogen protein conversion factor of 5.46. Peanut was incurred into either a chocolate dessert matrix based on that previously used in the EuroPrevall project for oral food challenges^[22,32] or a gluten-containing cookie matrix (Tables S2–S4, Supporting Information). The chocolate dessert matrix was reconstituted with 70% (v/w) water prior to use and contained 4% (w/w) peanut flour whilst the cookies contained 20.4% (w/w) peanut flour.

Peanut Allergic Patient Samples: Human serum and plasma samples from peanut-allergic patients ($n = 11$) were obtained from Manchester Respiratory and Allergy Biobank (ManARTS) which is funded by the National Institute for Health Research. Peanut allergy was assessed based on a positive skin prick test and peanut specific IgE together with a clinical history consistent with IgE-mediated peanut allergy. Additional plasma samples from peanut allergic patients were purchased from PlasmaLab International (Everett, USA). The characteristic and clinical information for the ManARTS patients is detailed in Tables S5 and S6, Supporting Information.

In Vitro Oral-Gastric-Duodenal Digestion: The in vitro digestion system mimicking the oral, gastric, and duodenal stages of digestion was adapted from that of Smith and co-workers^[28] with the gastric digestion also being performed at pH 6.5 to mimic intra-gastric pH observed in individuals taking anti-acids. In order to mimic conditions found in vivo the quantities of enzymes and volumes added to each of the foods was adjusted based on their nutritional composition, the level of proteases being adjusted to take account of the total protein content (Figure S1 and Table S4, Supporting Information). Each digestion was performed in triplicate together with a control sample (GU) which comprised a chew sample to which only SGF was added without pepsin. Digested samples were centrifuged at $16\,000 \times g$ for 20 min at 4 °C and the supernatant (soluble fraction) and pellet (insoluble fraction) collected and stored at –20 °C until required for further analysis.

SDS-PAGE and Immunoblotting: Prior to SDS-PAGE analysis, food samples (10 mg) or pellets from digested samples were extracted in 7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 50 mM DTT, 50 mM Tris-HCl, pH 8.8 at 60 °C in a sonicating water bath with intermittent (3×5 min) vortex mixing. After centrifugation at $16\,000 \times g$ for 30 min at 20 °C, the supernatant was removed. Samples were prepared for SDS-PAGE by adding NuPAGE LDS buffer (25 µL) to either 75 µL of pellet extract, or 50 µL of the soluble fraction of digestion samples; a further 25 µL of 200 mM DTT was also added to the latter. Samples were then heated to 90 °C for 5 min and 10 µL per well of each sample loaded on a 4–12% gradient NuPAGE gel and analyzed as previously described.^[34] Unstained SDS-PAGE gels were electro-blotted onto nitrocellulose membrane using a semidry blotting system (Bio-Rad Laboratories, Hercules, CA, USA) as previously described.^[34] Blots were developed as previously described using either animal antibodies,^[32] or human serum IgE.^[42]

IgE Immunoassay: Time points from the oral, oral-gastric, and oral-gastric-duodenal digestions of the peanut-containing matrices were selected based on their electrophoretic profiles. In addition, selected digestion time-points for matrices without peanut were used as negative controls. Corresponding soluble fractions were analyzed for Ara h 1-, Ara h 2-, and Ara h 6-related IgE immunoreactivity using an IgE capture inhibition assay.^[43,44] The assay is described in more detail in Section S1.2, Supporting Information.

Statistical Analysis: Statistical analyses were performed using GraphPad Prism 7.01 (GraphPad software, San Diego, CA, USA) or XLSTAT (version 2016.02.2744, Addinsoft).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

A.B. and C.A.C. prepared the food matrices, F.S. developed the detailed digestion methods and R.S. the IgE immunoblotting and histamine release methodology. A.S. and A.S.-J. undertook clinical evaluation of patients recruited in to the ManARTS tissue bank. H.R. executed all the experiments described apart from IgE immunoassays, which were undertaken by H.B. I.B., P.P., C.M., K.A.P., and W.X. supervised the work. H.R., I.B., and K.A.P. contributed to drafting the manuscript, which was written by C.M. All authors have reviewed and commented on the manuscript.

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