

ORIGINAL PAPER

Quantification of Ara h 1 in peanuts: why roasting makes a difference

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Experimental
Allergy

Summary

Background Increased allergenicity of roasted vs. raw peanut has been reported by showing higher IgE binding to roasted peanut extracts.

Objective To study the effect of roasting on Ara h 1 quantification in peanut using a specific monoclonal antibody-based ELISA, and to compare the Ara h 1 content from different kernel size peanuts from four runner cultivars.

Methods Raw or oven-roasted (177 °C for 5–30 min) runner peanuts were crushed and extracted at 60 °C. Inhibition ELISA was used to study binding of Ara h 1 purified from raw or roasted peanut. Runner peanuts of four different cultivars were collected, shelled, sized and roasted for 15 min at 177 °C. Ara h 1 in the extracts was compared by ELISA.

Results Ara h 1 levels were up to 22-fold higher in roasted than in raw peanuts (820 vs. 37 µg/mL, in a representative experiment) with an Ara h 1 peak at 10–15 min of roasting. Inhibition ELISA indicated that this increase was not due to conformational changes in the Ara h 1 monoclonal antibody epitopes. Ara h 1 was found at lower levels in number 1 than in jumbo- and medium-sized peanuts, and no differences were found among cultivars.

Conclusion These results suggest that roasting increases the efficiency of Ara h 1 extraction, and/or that the monoclonal antibody binding epitopes were more accessible in roasted peanut. Expression of Ara h 1 is associated with peanut maturity.

Keywords allergen quantification, food allergy, immunoassay, peanut allergen

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Introduction

Peanut allergy is an important health problem in the United States, affecting approximately 1.2% children [1]. This prevalence is much higher in Chinese-American than in Chinese populations, despite similar consumption rates of peanuts in the United States and China [2]. Differences in cooking methods suggested that roasting could contribute to the potency of peanut allergens. Differences in sensitization rates in the United States and China could be explained by consumption of roasted peanut in the United States, whereas in China and other Asian countries, peanuts are usually consumed boiled or fried [2]. Thermal processing may alter the allergenicity of foods by changing their susceptibility to digestion, altering the allergen tertiary structure, or destroying or creating new IgE-binding epitopes [3, 4]. Increased IgE binding to peanut allergens (Ara h 1, Ara h 2 and Ara h 3) from roasted peanuts has been reported, and IgE binding to roasted peanuts extracts was increased by 90-fold when compared

with raw peanut extracts [2, 5–7]. However, the reason(s) for this effect have not been proven. The observed increase of Ara h 1 could be due to: (a) the presence of more Ara h 1 in the extract due to increased efficiency of allergen extraction from roasted peanut, (b) an increased affinity of Ara h 1 from roasted peanut for IgE antibody, (c) a greater accessibility of IgE-binding epitopes from roasted peanuts or (d) increased sensitization to Ara h 1 from roasted peanut due to resistance to digestion. Published reports on the effects of roasting on antibody binding are contradictory. Earlier studies reported that thermal denaturation of peanut extracts did not affect IgE- or IgG-binding activity to Ara h 1 or Ara h 2 enriched fractions [8]. These studies suggest that roasting increases the quantity of allergen available for IgE (or IgG) antibody binding, rather than altering its affinity or epitope specificity. More recently, no significant differences in affinity were found between IgE and Ara h 1 purified from peanuts heated at different temperatures [9]. On the other hand, increased allergenicity of roasted peanut and an alteration

of Ara h 1 and Ara h 2 IgE binding epitopes by roasting that would manifest in an increased IgE-binding to the allergens have been proposed [10, 11].

In the present study, the effect of roasting on quantification of Ara h 1 was compared using a monoclonal antibody (mAb)-based ELISA [12]. The effect of roasting on mAb-defined epitopes was compared by inhibition ELISA. The ELISA was also used to study whether Ara h 1 levels per gram of peanut increased with peanut maturation (instead of Ara h 1 concentration been kept the same as peanut grows and matures), as suggested by a previous report of a 62.5 kDa protein peak, most likely Ara h 1, that increased in size as the peanut matured [13].

Materials and methods

Peanut samples

Thirty runner peanut samples were collected from four different cultivars (AT-201, C-99R, Georgia Green and ViruGard) grown in the same field using conventional, non-irrigated production practices. Peanuts were harvested at the optimum harvest date based on the hull-scraper method of maturity determination [14]. Samples were shelled and kernels were sized into commercial size categories [15]. To sort the peanuts into the commercial size categories for runner type peanuts, they were passed over a series of screens with slots 19 mm long and of a specified width: a jumbo-sized peanut rides a screen with a slot width of 8.3 mm; medium-sized peanut, slot width 7.1 mm; number 1-sized peanut, slot width 6.4 mm.

Effect of roasting peanuts on Ara h 1 measurement

Jumbo, AT-201, runner peanuts were oven-roasted at 177 °C (350 °F), a conventional temperature for roasting peanuts, for 5, 10, 15, 20, 25 and 30 min (~12 g peanut per condition) [16, 17]. Raw and roasted peanuts (2 or 3 g) were crushed in a mortar and pestle and extracted at 60 °C with phosphate-buffered saline (PBS), 1M NaCl, pH 7.4 and 1% non-fat dry milk. Peanut extracts were allowed to stand for 10 min, and the supernatant was centrifuged at 16 000 g for 5 min at room temperature. The supernatants were further centrifuged at 16 000 g for 5 min, and frozen at -20 °C. Ara h 1 was measured using mAb ELISA with different combinations of anti-Ara h 1 mAb for coating and detection of the allergen: coating with 2F7 mAb and detection with biotinylated 2F7 mAb or 2C12 mAb, or coating with 2C12 mAb and detection with biotinylated 2F7 mAb¹². Total protein content was measured by Advanced Protein Assay (Cytoskeleton, Denver, CO, USA).

To compare Ara h 1 content in peanut from different kernel sizes, peanuts of different sizes from four cultivars were simultaneously oven-roasted for 15 min at 177 °C in two batches: (i) 12 samples including three kernel sizes of

each of the four cultivars, (ii) two groups of nine samples each corresponding to three cultivars AT-201, C-99R and Georgian Green, and three kernel sizes per cultivar. Peanuts were extracted as described above, and Ara h 1 was measured by ELISA.

Immunodiffusion of Ara h 1 from peanut roasted at different times

An Ouchterlony assay was performed on a 1% agarose in PBS gel using extracts from peanut roasted at different times and a rabbit anti-Ara h 1 polyclonal antibody. Samples (30 µL) were loaded in wells around a central well that contained the antibody (80 µL). The gel was incubated overnight at 4 °C.

Ara h 1 purification

Runner peanuts (~50 g) from AT-201 variety, jumbo size, raw or roasted at 177 °C for 15 min, were crushed using mortar and pestle until a fine paste was obtained. In a fume hood, peanut paste was dissolved in pre-cooled acetone (4 °C) and suspended in a Buchner funnel over Whatman #1 filter paper (Whatman International Ltd., Maidstone, UK). The peanut paste was washed with 20 × w/v cold acetone and dried overnight at room temperature. Dried peanut powder was extracted for 30 min in 1 L PBS, 1 M NaCl, at 60 °C. The extract was centrifuged (14 000 g for 15 min at 4 °C), and the supernatant was sterile-filtered and stored at 4 °C.

Ara h 1 was purified from peanut extract through a 2F7 mAb affinity column equilibrated with PBS. Bound Ara h 1 was eluted with 0.1 M glycine, 150 mM NaCl, pH 2.5, and eluted samples were neutralized with 2 M Tris, pH 8.0. Purified Ara h 1 was concentrated and dialyzed against PBS.

Inhibition of monoclonal antibody binding to Ara h 1 with Ara h 1 from raw and roasted peanut

Two inhibition ELISA were performed to test the effect of roasting on inhibition of 2C12 and 2F7 mAb binding to Ara h 1. These assays were a modification of the Ara h 1 ELISA reported previously [12]. To test inhibition of mAb 2F7 binding to Ara h 1, microtitre plates were coated with 200 ng/well mAb 2C12 in 0.05 M carbonate-bicarbonate buffer, pH 9.6 overnight at 4 °C. Plates were incubated for 1 h with 2 µg/mL Ara h 1 from roasted peanuts, and for ~2 h with biotinylated 2F7 mAb (1:1000) that had previously been pre-incubated for ~2.5 h with decreasing concentrations of Ara h 1 purified from raw or roasted peanuts (doubling dilutions from 50 µg/mL to 49 ng/mL) (Fig. 1, top). Plates were incubated with streptavidin-peroxidase and color was developed by using ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid))

substrate. Absorbance was read at 405 nm after 10–15 min using a spectrophotometer [12].

To test inhibition of mAb 2C12 binding to Ara h 1, microtitre plates were coated with 1 µg/well mAb 2F7. Plates were incubated with 2 µg/mL Ara h 1 purified from either raw (experiment 1) or roasted (experiment 2) peanut. Plates were incubated for 30 min with biotinylated 2C12 (1 : 10 000) that had previously been pre-incubated for ~2.5 h with decreasing concentrations of Ara h 1 purified from raw or roasted peanuts (doubling dilutions starting at 165 and 1850 µg/mL of Ara h 1 from raw and roasted peanut, respectively) for each of the two experiments (Fig. 1, bottom).

A Fel d 1 inhibition curve was used as a control, following the first protocol described above. Microtitre plates were coated with 100 ng/well mAb 6F9 overnight at 4 °C, followed by 1 h incubation with 80 ng/mL rFel d 1, and 30 min incubation with biotinylated 3E4 mAb (1 : 1000) that had previously been pre-incubated for ~2.5 h with decreasing concentrations of rFel d 1.

Statistical analysis

Data were analysed using PROC GLM (SAS, V8, The SAS Institute, Cary, NC, USA) for significant differences among

means using cultivar and kernel size as independent variables.

Results

Effect of roasting time on Ara h 1 recovery

Ara h 1 was measured in extracts of peanut roasted for different times. Increasing amounts of Ara h 1 were recovered from peanuts roasted for up to 10–15 min, whereas decreasing amounts of Ara h 1 were measured in peanuts roasted for 20, 25 and 30 min. The Ara h 1 peak was found in peanuts roasted for 10–15 min with values up to 22-fold higher in roasted than in raw peanuts (820 and 27 µg/mL for peanut roasted for 15 min or for raw peanut, respectively) (Fig. 2a). The ELISA curves for measurable Ara h 1 were parallel (Fig. 2b). Curves of Ara h 1 measured using monoclonal antibody ELISA with three different combinations of mAb for coating and detection, vs. roasting time had a similar shape, with a peak around 15 min of roasting (data not shown). The Ara h 1 peak coincided with a reduction of 33–47% of the total protein measured in peanut extracts at 10–15 min of roasting. The total protein in peanut extracts was steadily

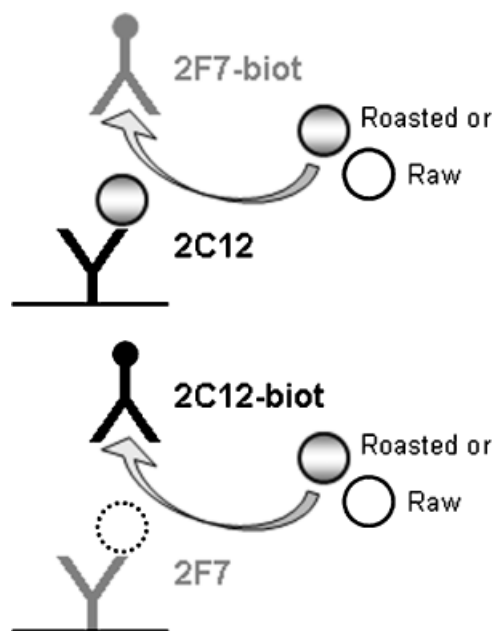


Fig. 1. Schematic representation of the ELISA inhibition assays. Top of the figure represents the assay to assess inhibition (arrow) of 2F7 biotinylated antibody binding to Ara h 1 from roasted peanut (2C12 mAb captured), by the inhibitor Ara h 1 from raw (empty circle) or roasted (filled circle) peanut (Fig. 3a). Bottom figure represents the assay to assess inhibition (arrow) of 2C12 biotinylated antibody binding to Ara h 1 (2F7 mAb captured) from roasted (Fig. 3b) or raw (Fig. 3c) peanut by the inhibitor Ara h 1 from raw or roasted peanut marked as above.

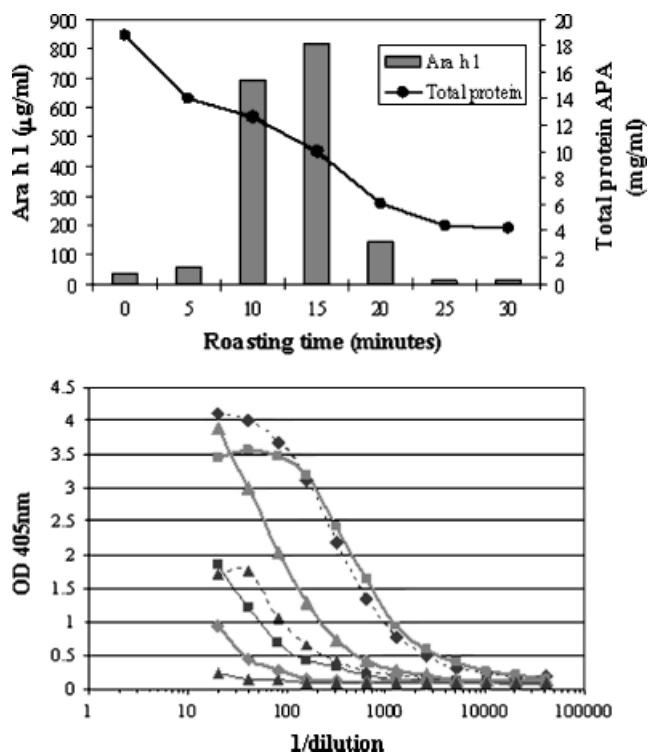


Fig. 2. Effect of roasting peanuts on Ara h 1 measurement. (a) Ara h 1 and total protein in extracts of peanut oven-roasted at 177 °C for different times. Ara h 1 was extracted and measured by mAb ELISA [12]. (b) ELISA curves of Ara h 1 from peanut roasted at different times: raw (—■—), 5 (—△—), 10 (—◆—), 15 (—□—), 20 (—▲—), 25 (—◇—) and 30 (—▲—) min. Data representing one of three different experiments.

reduced by up to 78% in peanuts roasted for 30 min (Fig. 2a). The Ouchterlony assay showed an excellent correlation between a positive value of Ara h 1 ELISA and immunoprecipitation. There was less immunoprecipitation at 20 min and none at 25–30 min.

Ara h 1 purification from raw and roasted peanuts

Ara h 1 was purified by affinity chromatography from extracts of raw and roasted runner peanuts (AT-201 variety, jumbo size). In keeping with the results that show increased recovery of Ara h 1 from roasted peanut, >3-fold more Ara h 1 was purified from roasted than from raw peanut. The purity of the Ara h 1 preparations was assessed by SDS-PAGE (data not shown). Ara h 1 had the expected molecular weight (63.5 kDa) and no significant difference was observed between Ara h 1 from raw and roasted peanut.

Inhibition enzyme linked immunosorbent assay experiments

The goal of the inhibition immunoassays was to investigate whether one or both mAb involved in the ELISA

showed differences in Ara h 1 binding between allergen purified from raw and from roasted peanut that could explain the peak of Ara h 1 measured in roasted peanut. The same binding of 2F7 for Ara h 1 from raw and roasted peanut was observed, as indicated by the inhibition curves of mAb 2F7 binding to Ara h 1 which had the same sigmoid shape for both inhibitors (Ara h 1 from raw and from roasted peanut) and the same IC₅₀ of ~3 µg/mL (Fig. 3a). This curve was comparable in shape with the control inhibition curve of mAb 3E4 binding to Fel d 1 with an IC₅₀ of ~10 µg/mL (Fig. 3d).

However, the 2C12 curves had a smaller slope than the curves of mAb 2F7 binding to Ara h 1, and spanned more than two orders of magnitude (Figs 3b and c). The inhibition curve of mAb 2C12 binding to Ara h 1 was displaced to the right when the inhibitor was Ara h 1 from roasted instead of raw peanut, indicating that 2C12 binds Ara h 1 from raw peanut with more affinity than the allergen from roasted peanut. The curve showed an initial increase in 2C12 mAb binding to ~120% when the inhibitor was Ara h 1 from roasted peanut. This increase was larger (~150%) when the binding of the mAb was to Ara h 1 from raw peanut instead of roasted (Figs 3b and c).

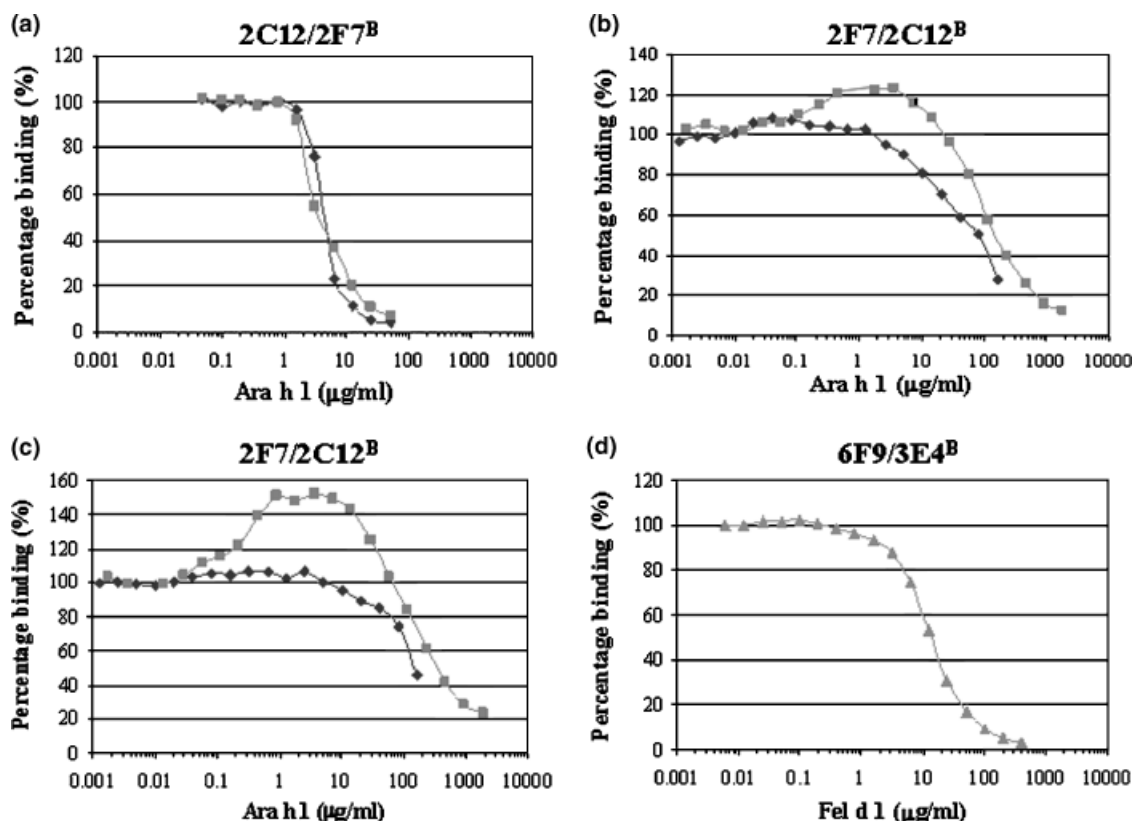


Fig. 3. Inhibition of mAb binding to Ara h 1. Inhibition curves A–C used Ara h 1 purified from raw (—◆—) and from roasted (—■—) peanut as inhibitors. In curve D the inhibitor is Fel d 1 (—▲—). (a) inhibition of mAb 2F7 binding to Ara h 1 from roasted peanut, (b) inhibition of mAb 2C12 binding to Ara h 1 from roasted peanut, (c) inhibition of mAb 2C12 binding to Ara h 1 from raw peanut and (d) inhibition of mAb 3E4 binding to Fel d 1. mAb combinations are indicated at the top of each plot as coating antibody/biotinylated detection antibody.

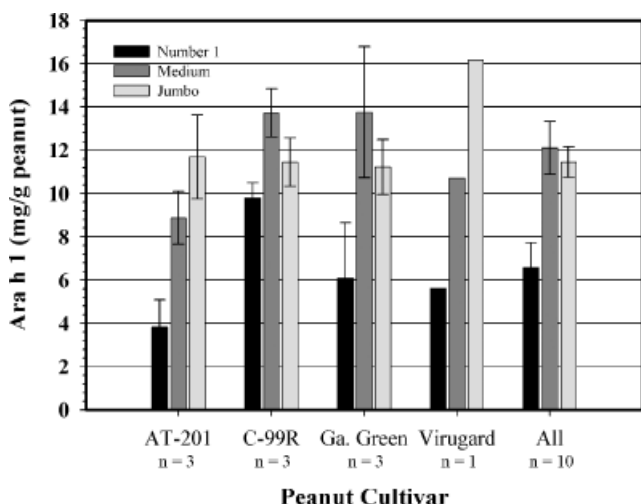


Fig. 4. Ara h 1 in different peanut cultivars and kernel size. Ara h 1 measured in runner peanuts of different cultivars (AT-201, C-99R, Georgia Green and Virugard) and kernel size: Number 1 (black), medium (dark grey) and jumbo (light grey). The number of samples of each cultivar is indicated (*n*). Right columns represent the average of Ara h 1 in the total samples for each kernel size. Values are means with the standard error of the mean.

The mAb 2F7 and 2C12 bound Ara h 1 differently, as shown by the overall shape of the inhibition curves, and the binding affinity was approximately 100-fold less for 2C12 than for 2F7, as shown by a displacement of the 2C12 curves to the right (Fig. 3).

Ara h 1 in different Runner peanut cultivars and kernel sizes

Results of Ara h 1 measured in different kernel size peanut from four Runner peanut cultivars are shown in Fig. 4. When Ara h 1 concentrations were averaged by kernel sizes across all cultivars (*n* = 30), significant differences were noted. The Ara h 1 concentration was significantly lower in the number 1-sized kernels than either the jumbo- or medium-sized kernels. However, no significant difference was detected between jumbo- or medium-sized kernels (Fig. 4). Within cultivar, there were no significant differences in the levels of Ara h 1 due to kernel size, except within the AT-201 cultivar, which showed similar results as above: the number 1-sized kernels had significantly less Ara h 1 than the jumbo-sized kernels but not the medium-sized kernels. There were no differences between the jumbo- and medium-sized kernels. No significant differences in Ara h 1 concentration due to cultivar were detected.

Discussion

We report an increase of Ara h 1 measured in roasted compared with raw peanut extracts, with a peak for

peanut roasted for 10–15 min, whereas the total peanut protein content decreased with roasting time. At higher roasting times (25–30 min), Ara h 1 conformational changes led to a loss of immuno-recognition by a polyclonal anti-Ara h 1 antibody in the Ouchterlony assay. The coincidence of the Ara h 1 peak with a steady decrease of total protein content indicates that many proteins in the peanut extract, unlike Ara h 1, are not heat stable. The Ara h 1 peak could be explained by an increase in the efficiency of Ara h 1 extraction from roasted peanut and/or a greater accessibility of the mAb-binding epitopes from roasted peanut. Another possible explanation, discarded by the ELISA inhibition experiments, is that heat-induced conformational changes in Ara h 1 would increase binding of the monoclonal antibodies used in the mAb ELISA (raised against Ara h 1 from raw peanut). The binding of mAb 2F7 to Ara h 1 was equally inhibited by Ara h 1 purified from raw or roasted peanut, suggesting that the 2F7 mAb epitope had not been altered by roasting. The inhibition curve had a sigmoid shape similar to the control Fel d 1. On the other hand, roasting modifies the 2C12 mAb binding epitope, decreasing the apparent affinity for Ara h 1, as shown by the displacement of the inhibition curve to the right when the inhibitor was Ara h 1 from roasted peanut. The shape of the curves, different for each inhibitor, shows an initial increase of binding when the inhibitor is Ara h 1 from roasted peanut. This effect could be related to the higher affinity of 2C12 for Ara h 1 from raw peanut and the presence of more mAb 2C12 available to bind captured allergen when the inhibitor is Ara h 1 from roasted peanut. If captured Ara h 1 is from raw peanut, more increase was observed than when is from roasted peanut (which may still contain some allergen less affected by roasting). In summary, the peak of Ara h 1 observed at 10–15 min of roasting is not due to an increased binding affinity of Ara h 1 for 2F7 and/or 2C12.

As the mAb 2F7-binding epitope was not altered by roasting, an ELISA using the mAb 2F7 for coating and detection was used to confirm that the increase of Ara h 1 extracted from peanut roasted for 10–15 min is independent of changes in the mAb binding epitopes. This assay also revealed that more than one binding site for the mAb 2F7 is present in Ara h 1, which could be explained by the trimeric nature of this allergen [18]. Therefore, the increase of Ara h 1 measured from roasted peanut extracts is due either to a higher yield of Ara h 1 extracted from roasted peanut or to an increase of epitope accessibility from roasted than raw peanut. This argument explains the results of studies reporting a 90 fold higher IgE-binding to roasted than raw peanut extracts, and that roasting may alter the sensitizing ability of peanut allergens [5, 10, 11]. In these studies, peanut extracts instead of purified allergens were used as inhibitors in a direct competitive ELISA [5]. Under these conditions, it is likely that the difference

observed between raw and roasted extracts is due to a different amount of Ara h 1 and/or different epitope accessibility in Ara h 1 from raw and roasted peanuts, as shown in the present study, and not to a change on IgE-binding epitopes.

Koppelman et al. [9] did not observe significant differences in the affinity of IgE for Ara h 1 purified from peanuts heated at different temperatures. The polyclonal nature of IgE antibodies used in this study would not allow distinction of binding affinity for raw and roasted peanut, if only one or few epitopes were affected by heat. They also reported that heat leads to a secondary more structured conformation of the protein with the formation of extended β -structures [9]. This change could affect the conformation of a specific epitope, such as the 2C12 mAb-binding epitope, without modifying the overall binding of the polyclonal IgE. The use of the mAb ELISA using the 2F7 mAb for capture and detection of the allergen, will allow Ara h 1 measurements independent of conformational changes in Ara h 1.

A linkage between peanut allergenicity and the processes of maturation, curing and roasting has been reported [7]. We studied the relationship between peanut maturity and Ara h 1 content, taking advantage of the fact that by recovering more Ara h 1 from roasted peanut, we can better assess differences in Ara h 1 concentration in different size kernels. The peanut plant, once starts fruiting, will generally add about two pods a day until environmental conditions trigger a shut down. The peanuts on an individual plant at the time of harvest have a range of maturity and size. Within a given commercial size category, there is a range of maturity, but generally, the larger size kernels have a higher percentage of mature kernels. Number 1-sized kernels typically have a higher percentage of immature kernels than do jumbo- or medium-sized kernels [14]. In this study, a higher Ara h 1 concentration was found in peanut samples of large kernel size. Therefore, higher Ara h 1 content is an indicator of kernel maturity. Additionally, no differences in Ara h 1 peanut content were found among cultivars of the tested market type runner. Koppelman et al. [19] found no differences among market types Valencia, Runner, Virginia and Valencia, but differences among cultivars, which are subsets of a particular market type, had not been investigated.

In summary, recovery of Ara h 1 was increased in shelled jumbo-size peanuts roasted for 10–15 min. The conditions tested emulate conventional oven roasting ('parching') that takes place in a 350°F oven during 15–20 min for shelled and 20–25 min for in-shell peanuts. ELISA inhibition assays indicated that this increase was independent of conformational changes in the Ara h 1 mAb epitopes. Rather, an increased efficiency of extraction from roasted vs. raw peanut and/or a higher accessibility of the mAb binding epitopes in roasted peanut is indicated. An ELISA is being developed

for Ara h 2, and similar studies will complete the analysis of the effect of roasting on the allergens involved in peanut allergy. Implications of the present results are that Ara h 1 exposure will be different for the same peanut weight ingested depending on the degree of roasting and kernel size. Therefore, sensitization levels will most likely depend on roasting and kernel size. Threshold studies of peanut exposure may require standardization of the peanut roasting conditions and size of the kernel to make the challenges used in clinical trials comparable.

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