
Characterization and improvement of apple extracts for the diagnosis of apple IgE-mediated allergy

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Background: The production process of reliable fruit extracts is not well established.

Objectives: To improve the overall quality of apple extracts by reducing protein loss during the manufacturing process and to evaluate the improved extracts using *in vivo* and *in vitro* experiments.

Methods: Two types of extracts were prepared from peels of Golden Delicious apples (*Malus domestica*). Extract A was extracted, 1:2 wt/vol, for 30 minutes at 4°C in 0.01M phosphate-buffered saline, and extract B was extracted, 1:2 wt/vol, in phosphate-buffered saline with 20% polyvinylpyrrolidone and 2-mmol/L EDTA. Both extracts were filtered, dialyzed in 3.5-kDa dialysis membranes, and lyophilized. The antigenic and allergenic profiles were analyzed using immunoblot and enzyme-linked immunosorbent assay. Nine patients clinically sensitive to apples and 12 controls underwent skin testing with both extracts.

Results: Extracts A and B had dry weight yields of 0.71% and 1.86% and protein contents of 104.6 and 257 µg/mg of freeze-dried material, respectively. A steady and progressive loss of protein, greater in extract A than in extract B, was detected at different intervals during the manufacturing process of both extracts. Extract B produced larger wheal sizes than extract A ($P = .008$). Enzyme-linked immunosorbent assay inhibition results confirmed that extract B had a greater inhibition capacity than extract A.

Conclusions: A progressive loss of protein content occurs during the manufacturing of apple extracts. Wheal sizes induced by extract B were significantly larger than those induced by extract A and prick-by-prick solutions. Extract B was also more potent *in vitro* than extract A.

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INTRODUCTION

Adverse reactions to fruits are an increasing cause of medical concern. However, in some cases, the efficacy of the diagnostic extracts is questionable.¹ False-negative results in the diagnosis of food allergy are mainly due to the instability, low protein content, and high proportion of sugars and phenolic compounds of food allergen extracts.² Owing to the increasing prevalence of food allergies, there is an emerging need to improve the overall extraction and conservation process of fruit extracts.

Apples are one of the most important fruits implicated in allergic symptoms in Mediterranean countries. In northern Europe, birch pollen induces many sensitizations, and more than 75% of these patients are sensitized to apples.^{3,4} However, in Mediterranean countries, sensitization to birch pollen is uncommon because birch trees are scarce. Therefore, allergy to apple is not related to birch pollen, and sensitization occurs primarily through ingestion.⁵ The allergen involved in

reactions to apple seems to be a 9-kDa protein that has been identified as a lipid transfer protein and is called Mal d 3.⁶

Although pollen extracts are well characterized and standardized, the quality of food extracts remains controversial. A recent study⁷ suggested that apple peel extracts prepared using improved methods produced wheal sizes comparable with those obtained using prick-by-prick methods and better than the commercial extracts. The availability of improved food extracts could also have a substantial impact on the *in vitro* diagnosis of food hypersensitivities. The standardization of food extracts is also an important prerequisite for future food immunotherapy trials.

The aims of this study were to monitor the production process of apple extracts, to improve the overall quality of these extracts by reducing protein loss during the manufacturing process, and to evaluate the improved extracts using *in vivo* and *in vitro* experiments. Patients from the Mediterranean coast of Spain (Alicante) were evaluated in this study.

MATERIALS AND METHODS

Patient Population

A total of 21 individuals were included in this study. All the participants were provided with a summary and a detailed explanation of the study before enrollment. Patients who agreed to participate signed informed consent forms.

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Nine patients (7 men and 2 women; mean $\pm$ SD age, 23.77 $\pm$ 8.3 years) had a clinical diagnosis of IgE-mediated allergy after eating apples.⁸ They all had symptoms in the oral and pharyngeal cavities (oral allergy syndrome [OAS]); none of them had systemic reactions after eating apples. Apple-allergic patients underwent an open food challenge with an apple to confirm the diagnosis of apple-induced OAS. All the patients had signs and symptoms suggesting apple-induced OAS after biting an apple. All these patients were also sensitized to peach and had clinical symptoms after direct contact with or ingestion of this fruit. Blood samples collected from all the patients were stored at -20°C .

Twelve individuals were used as controls (4 men and 8 women; mean $\pm$ SD age, 32.1 $\pm$ 14.5 years); 5 of these individuals (42%) were sensitized to *Olea europaea*, grasses, *Salsola kali*, *Artemisia vulgaris*, *Parietaria judaica*, *Dermatophagoides* species, and *Alternaria alternata* in variable degrees. The remaining 7 individuals were nonallergic and had negative skin test results.

Extract Manufacturing and Protein Purification

Golden Delicious apples (*Malus domestica*) were bought at a local market and carefully peeled; the peel was used to prepare the extracts. Two types of extracts were prepared. For extract A, 64.5 g of peels was homogenized and extracted for 30 minutes (1:2 wt/vol) in 0.01M phosphate-buffered saline (PBS), pH 7.4. Extract B was prepared according to the method of Björkstén et al⁹ incorporating minor modifications: 52.6 g of peels was extracted for 30 minutes in 0.01M PBS with 20% polyvinylpyrrolidone and 2-mmol/L EDTA. Both supernatants were collected (separately), sterile filtered using 0.22- μm filters, dialyzed against bi-distilled water, and freeze-dried.

The protein content was measured in both extracts using the Lowry-Biuret method (Sigma-Aldrich Corp, St Louis, MO); the results are expressed as percentage of protein per milligram of dry weight. Furthermore, samples were collected after homogenization, extraction, centrifugation, dialysis, and filtration, and the protein content was measured. The results are expressed as milligrams of protein per gram of raw material.

Skin Prick Tests

Skin prick tests were prepared at a concentration of 5 mg of freeze-dried material per milliliter. All 21 individuals underwent skin prick testing, in duplicate, using both prick test solutions (extracts A and B) and using fresh apple peel following the prick-by-prick method described by Dreborg and Foucard.¹⁰ After 20 minutes, patients with a wheal diameter greater than 3 mm were considered positive according to the guidelines of the European Academy of Allergology and Clinical Immunology.¹¹

Direct Enzyme-Linked Immunosorbent Assay

Serum samples from the 9 apple-positive individuals were tested using direct enzyme-linked immunosorbent assay (ELISA) to determine specific IgE levels to both apple peel extracts and *Betula verrucosa*. Briefly, 10 $\mu\text{g/mL}$ of material

was dissolved in carbonate/bicarbonate buffer, pH 9.6, and coated onto plastic microtiter plates (Immulon IV; Dynex Technologies, Chantilly, VA). Each serum sample was diluted 1:2 vol/vol in 0.01M PBS and incubated (100 μL) for 2 hours in the wells. The plates were washed and incubated for 2 hours in anti-human IgE conjugated with peroxidase. After 5 washes, the reaction was developed for 30 minutes and then stopped with sulfuric acid 1 N. Three individual serum samples from nonallergic patients were used as negative controls. Optical densities (ODs) 3 times the mean value of the negative controls or greater were considered positive. A serum pool was prepared with serum samples from 10 patients who had detectable specific IgE to apple.

ELISA Inhibition

Plastic microtiter plates (Immulon IV) were coated with extract B (20 $\mu\text{g/mL}$). Several dilutions were made from extracts A and B. Each dilution was incubated in the serum pool, diluted at a ratio of 1:2, for 2 hours. Afterward, the dilutions were incubated for 2 more hours in the coated wells. The allergenic potency was calculated and is expressed as micrograms needed to reach 50% inhibition.

Sodium Dodecyl Sulfate-Polyacrylamide

Gel Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was used to determinate the protein profiles of both extracts. Samples were run in SDS-PAGE gel with 2.67% crosslinker and 15% acrylamide as described by Laemmli.¹² Samples were diluted in sample buffer containing β -mercaptoethanol and denatured at 100°C for 10 minutes and centrifuged for 1 minute at 10,000 rpm; 200 μg of freeze-dried material of extracts A and B was loaded per lane. After SDS-PAGE, the gel was fixed for 45 minutes and silver stained (BioRad Laboratories, Hercules, CA).

Immunoblots

Separated bands of extract B were electrophoretically transferred to P-Immobilon membrane (Millipore, Bedford, MA). After transfer, the membrane was dried for 4 hours, incubated overnight in the 9 individual serum samples diluted in 0.01M PBS, 2% Tween, and a serum pool of *B verrucosa*-allergic individuals. Specific IgE binding was detected by using peroxidase-conjugated monoclonal anti-human IgE (Ingenasa, Madrid, Spain).

Statistical Analysis

The mean $\pm$ SD and median wheal sizes induced by both extracts and the fresh fruit were calculated. The Shapiro-Wilks test was used to determine the normal distribution, and the Wilcoxon test was used to evaluate the differences in the wheal sizes induced by the 3 prick tests.¹³

RESULTS

Extract Manufacturing

The yield of the manufacturing process for extract A was 0.71%, obtaining 1.065 g of dry weight, and for extract B was

1.86%, obtaining 2.79 g of dry weight. The protein content of extracts A and B were 104.6 and 257 $\mu\text{g}/\text{mg}$ of freeze-dried material, respectively. The protein content was also measured at 5 different intervals in the manufacturing process (Figure 1). A progressive decrease in protein content at each step of the process was detected. Extract A had less protein than extract B at all intervals.

Skin Prick Tests

A positive skin test reaction was induced by extract A in 89% of the patients, by extract B in 100%, and by prick-by-prick solutions in 78%. The mean $\pm$ SD wheal size obtained with extract A was $20.62 \pm 16.33 \text{ mm}^2$ (median, 11 mm^2), with extract B was $37.78 \pm 21.38 \text{ mm}^2$ (median, 41 mm^2), and by prick-by-prick solutions was $23.86 \pm 20.51 \text{ mm}^2$ (median, 26 mm^2) (Fig 2). The statistical analysis (Wilcoxon test) demonstrated a significant difference between extracts A and B ($P = .008$) and between extract B and prick-by-prick solutions ($P = .01$) but not between extract A and prick-by-prick solutions.

Direct ELISA

The presence of specific IgE against both apple peel extracts was evaluated in the serum samples of the 9 apple-positive individuals; 33% were positive against extract A and 67% against extract B. The mean OD of the positive serum samples for extract A was 0.280 and for extract B was 0.352. This difference is statistically significant ($P = .02$, Wilcoxon test). The mean OD of the negative control was 0.055. Only 1 of the 9 patients had detectable levels of specific IgE to *B verrucosa* (OD = 0.75).

ELISA Inhibition

The allergenic potencies of extracts A and B were compared using ELISA inhibition experiments. The 2 curves had a correlation coefficient (r value) greater than 0.95. The results revealed that extract B was slightly stronger than extract A: 4.42 μg of protein was necessary to reach 50% inhibition for extract B and 5.68 μg of protein was necessary for extract A (Fig 3). The curves paralleled each other (t test).

SDS-PAGE and Immunoblot

The protein profiles of both apple peel extracts are shown in Figure 4A. Several bands were detected in a molecular weight range of 9 to 65 kDa. Mal d 3 was the most prominent band recognized by the serum pool in both extracts (Fig 4B).

DISCUSSION

Several studies^{14,15} have demonstrated great variability in the allergenic potency of commercial fruit extracts, including apples and peaches. In the present study, we describe the production process of 2 different apple peel extracts and the consequences that improvements in the manufacturing process have on the in vivo and in vitro allergenicity of these extracts. We decided to investigate an apple peel extract because apple peels have greater protein content than the pulp and based on previous observations with peach, in which we confirmed that the content of Pru p 3, homologous to Mal d 3, was approximately 7 times greater in peel than in pulp.¹⁶

The quality of fruit extracts remains controversial. It has been suggested that the allergenic activity decreases during the manufacturing of the extracts as a result of interactions

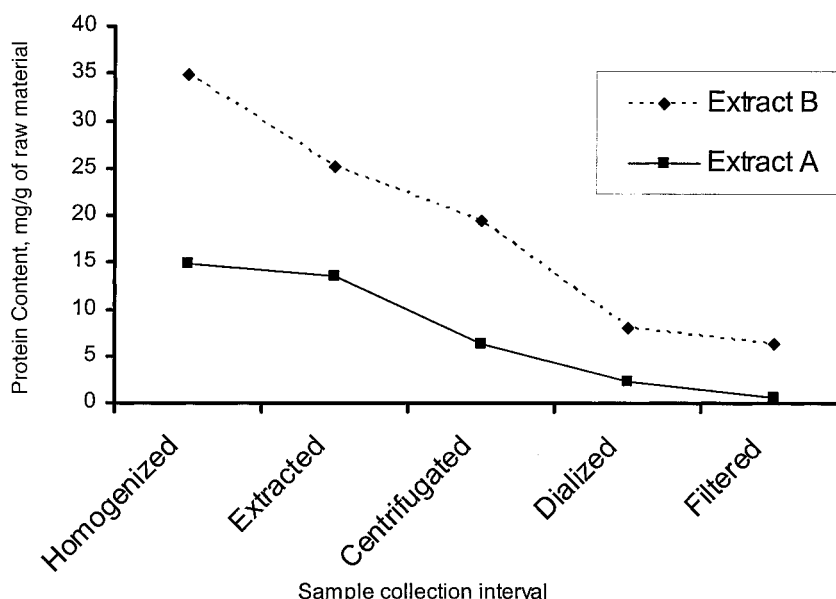


Figure 1. Protein content in both extracts at each step of the manufacturing process.

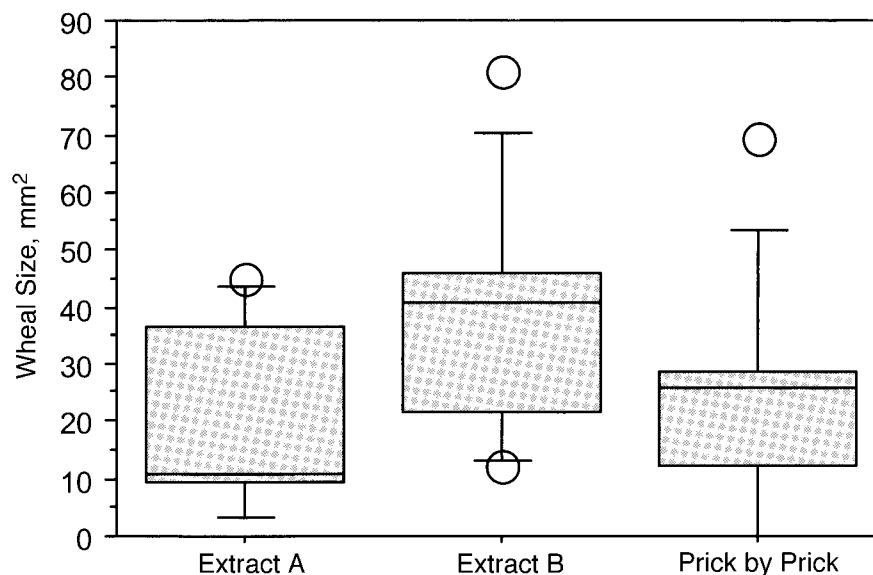


Figure 2. Box plot of the wheal sizes induced by extract A (phosphate-buffered saline), extract B (phosphate-buffered saline with polyvinylpyrrolidone), and prick-by-prick testing. The horizontal lines represent the 10th, 25th, 50th, 75th, and 90th percentiles; all values above the 90th and below the 10th percentiles are depicted as white circles.

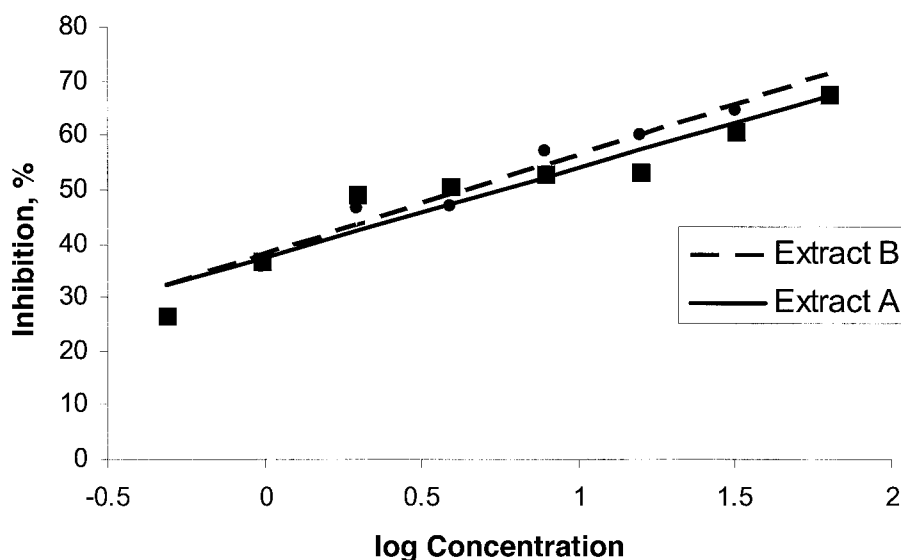


Figure 3. Enzyme-linked immunosorbent assay inhibition experiments between extracts A and B. Experiments were conducted using a pool of serum samples.

between proteins and phenolic compounds. The end result may be an extract of poor quality and little efficacy for diagnosis. Polyphenol oxidase and different proteolytic enzymes are the responsible agents implicated in the browning of tissue plants, causing oxidation and polymerization of phenolic compounds.¹⁷ At the end, proteins may be destroyed, and, therefore, the diagnostic capacity of the extract is significantly reduced. Consequently, conventional extraction methods during extensive periods in PBS are not suitable

because allergens may be destroyed.¹⁸ An alternative solution is to protect the proteins with antioxidants and protective substances that enhance the protein yield.

We demonstrated that the protein content declines during the filtration process, reducing the overall protein yield of the extract. Previous studies have not evaluated this reduction of protein content occurring during the manufacturing of food extracts, which could be in part responsible for the overall decrease in the potency of fruit extracts. In this study, we

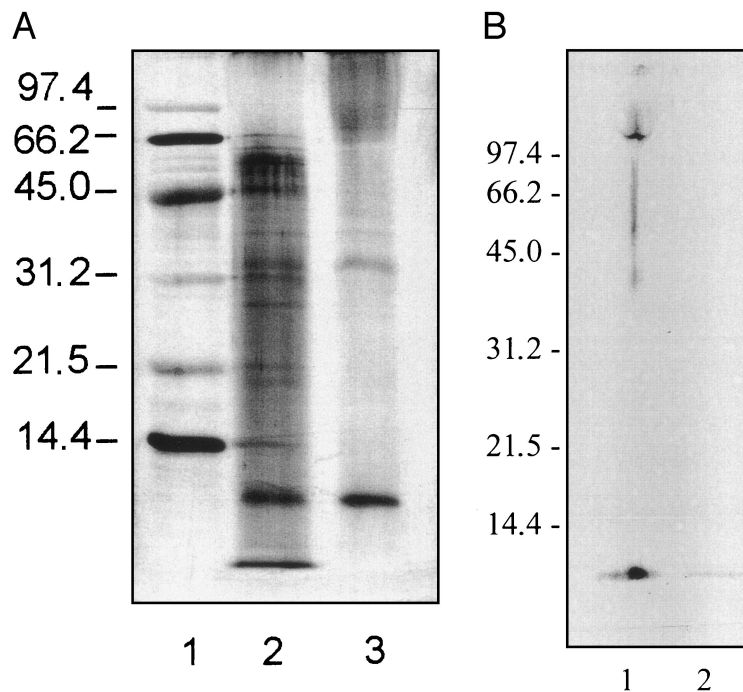


Figure 4. A, Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis of the apple extracts. Lane 1 shows molecular weight standards; lane 2, extract B (200 µg per lane); and lane 3, extract A (200 µg per lane). B, Western blot analysis of the apple extracts. Lane 1 shows extract B; lane 2, extract A.

confirmed a progressive and steady decline in protein content at different stages of the production process. This loss, which does not occur in prick-by-prick preparations, could explain the better diagnosis capacity that these extracts have demonstrated so far.

It is evident that standardized extraction procedures for fruits need to be improved. However, is there a real benefit in using fruit extracts compared with using fresh fruits when performing skin prick tests? One important consideration to foster the use of standardized fruit extracts instead of fresh fruits is that certain fruits are not always available. For in vitro tests, these new extracts may be superior, but the reliability of the new extracts for in vivo diagnostics had to be tested.

The extract that incorporated protective components into the extraction buffer had a better overall protein yield and was more allergenic in vivo and in vitro. To validate the extracts in vivo, we compared the wheal sizes obtained using the prick-by-prick method and those obtained using extracts A and B. Extract B, at 5 mg/mL, identified all patients and induced greater skin test reactions than prick-by-prick methods and extract A. The results may be due to a greater concentration of allergen and protein per milligram of freeze-dried material in extract B than in the 2 other extracts, including prick-by-prick preparations. No irritant reaction was observed in atopic and nonatopic patients used as controls. The differences between extract A and prick-by-prick testing were not statistically significant, which suggests that in some cases the concentrations in prick-by-prick solutions are not high enough to

produce reliable skin test results, and higher allergen concentrations may be needed. Similar results were obtained by Cuesta-Herranz et al¹⁵ analyzing peach extracts. The wheal size induced using the maximum concentration of the peach extract was greater than that induced by fresh extract.¹⁵ It should be taken into consideration that in most cases, prick-by-prick skin tests in apple-sensitized patients are conducted using pulp extracts, which may not contain an adequate allergen concentration. The specific IgE inhibition assays also confirmed that extract B was more potent than extract A. The parallelism test demonstrated that the lines were parallel, suggesting that both extracts were similar qualitatively.

Apple allergy is one of the most common causes of fruit sensitization in Mediterranean countries. The antigenic profile of the apple peel extract revealed that the most prominent band in extract B corresponds to an allergen of 9 kDa (Mal d 3), which is implicated in the OAS induced by apples in patients living in Mediterranean countries. In Spain, and based on our experiments, apple sensitization seems not to be related to birch sensitization. In the present study, only 1 patient had detectable levels of specific IgE to *B verrucosa*, confirming the hypothesis that in this population, the primary sensitization is due to apple and not to birch pollen, as in northern and central Europe.¹⁹ Another salient point is that all apple-sensitized individuals were also sensitized to peach, suggesting a great degree of allergen cross-reactivity and corroborating that in Mediterranean countries, sensitization to apple and peach is mediated by specific IgE to lipid transfer protein.

In conclusion, we identified a progressive loss of material and protein content during the manufacturing of apple extracts. Wheal sizes induced by the improved extract were significantly greater than those induced by an extract prepared using PBS and similar to those obtained using prick-by-prick methods. A concentration of 5 mg of freeze-dried material per milliliter of an improved extract induces greater wheal sizes than prick-by-prick tests.

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