

Allergenicity of 10 different apple varieties

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Background: More than 100 apple varieties are consumed worldwide. However, the allergenic composition of most apple varieties has not been fully characterized.

Objective: To analyze the antigenic and allergenic profiles of 10 different commercially available apple varieties.

Methods: Golden, Golden Perlim, Reineta, Reineta Parda, Fuji, Verdedoncella, Granny-Smith, Pink Lady, Royal Gala, and Starking apple varieties were carefully peeled. Peels were individually extracted and centrifuged, and the supernatants were collected, dialyzed, filtered, frozen, and freeze-dried. The extracts were characterized using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblots. Protein content was measured using the Lowry-Biuret method. Twenty-two Spanish patients with oral allergy syndrome after apple ingestion were included in the study. Specific IgE to all apple varieties and birch pollen was evaluated. Eleven patients underwent skin prick testing using extracts of Golden, Starking, and Reineta Parda varieties to determine differences in their *in vivo* allergenicity.

Results: The antigenic profile of the 10 varieties showed differences using sodium dodecyl sulfate–polyacrylamide gel electrophoresis. A 9-kDa band, which could correspond to Mal d 3, was visualized in all the extracts. The Starking variety had the highest protein content. Higher specific IgE values were also obtained for the Starking variety, and the lowest values were for Reineta Parda and Royal Gala. The largest wheals were obtained in all patients with the Starking variety, and the smallest with Reineta Parda. Fourteen patients (64%) recognized Mal d 3 in the Golden variety using immunoblots; 6 patients (27%), who also had specific IgE to *Betula*, recognized bands with higher molecular weights.

Conclusions: We demonstrated differences in the antigenic and allergenic profiles of the analyzed varieties and a significant variation in Mal d 3 content.

Ann Allergy Asthma Immunol. 2006;96:564–570.

INTRODUCTION

Apple, *Malus domestica*, belongs to the family Rosaceae and is one of the most widely cultivated tree fruits. The easy adaptation of the apple tree to different climatic conditions and soils and its resistance to low temperatures have allowed their cultivation in different climatic regions. It is a highly consumed fruit in the world, and several varieties are commercialized. More than 1,000 apple varieties have been described; however, only a small proportion is usually consumed.

Apples, along with peaches, are the most important fruits implicated in oral allergy syndrome in Mediterranean countries. The oral allergy syndrome is characterized by IgE-mediated symptoms that occur mainly at the mucosa of the lips, tongue, and pharynx after ingestion. Systemic reactions, including anaphylaxis, may occur in apple-allergic patients without related pollinosis. More than 75% of individuals with birch pollen sensitivity, which is frequent in some northern European countries, are sensitized to apples as a consequence of the cross-reactivity.^{1,2} 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 in this area, and sensitization occurs primarily through ingestion.³ In these cases, it has been demonstrated

that the allergens implicated belong to the group of lipid transfer proteins (LTPs) present in the Rosaceae family. In apple, LTP is a 9-kDa protein termed *Mal d 3*.⁴ Other allergens have also been characterized, such as Mal d 1 (Bet v 1 homologous),⁵ Mal d 2 (thaumatin-like protein),⁶ and Mal d 4 (profilin)² (Swiss-Prot accession No. Q9XF42).

It has been demonstrated that there are important differences in the allergenic potency of peel and pulp extracts in Rosaceae.⁷ The fruit variety and ripening stage could also be factors to consider.⁸ Previous studies conducted in olive pollen have demonstrated that the allergenicity could depend on the selected variety.⁹ It is also known that fruit extracts contain a low concentration of proteins and that the allergens are frequently unstable; therefore, optimal extraction conditions should be used.^{10,11} Selection of the correct variety and use of an optimized method of extraction are important points to consider when preparing extracts for diagnostic testing, reducing the risk of false-negative results. The aims of this study were to analyze the antigenic and allergenic profiles of 10 apple varieties, to study the allergenic differences among these varieties, and to determine Mal d 3 (LTP) content.

MATERIALS AND METHODS

Extract Manufacturing

Ten different apple varieties, purchased at a local market, were used in the study: Royal Gala, Pink Lady, Starking, Granny-Smith, Verdedoncella, Fuji, Golden, Golden Perlim, Reineta, and Reineta Parda (Fig 1). Apple extracts were prepared following previously described methods.¹⁰ Briefly,

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Accepted for publication in revised form September 22, 2005.

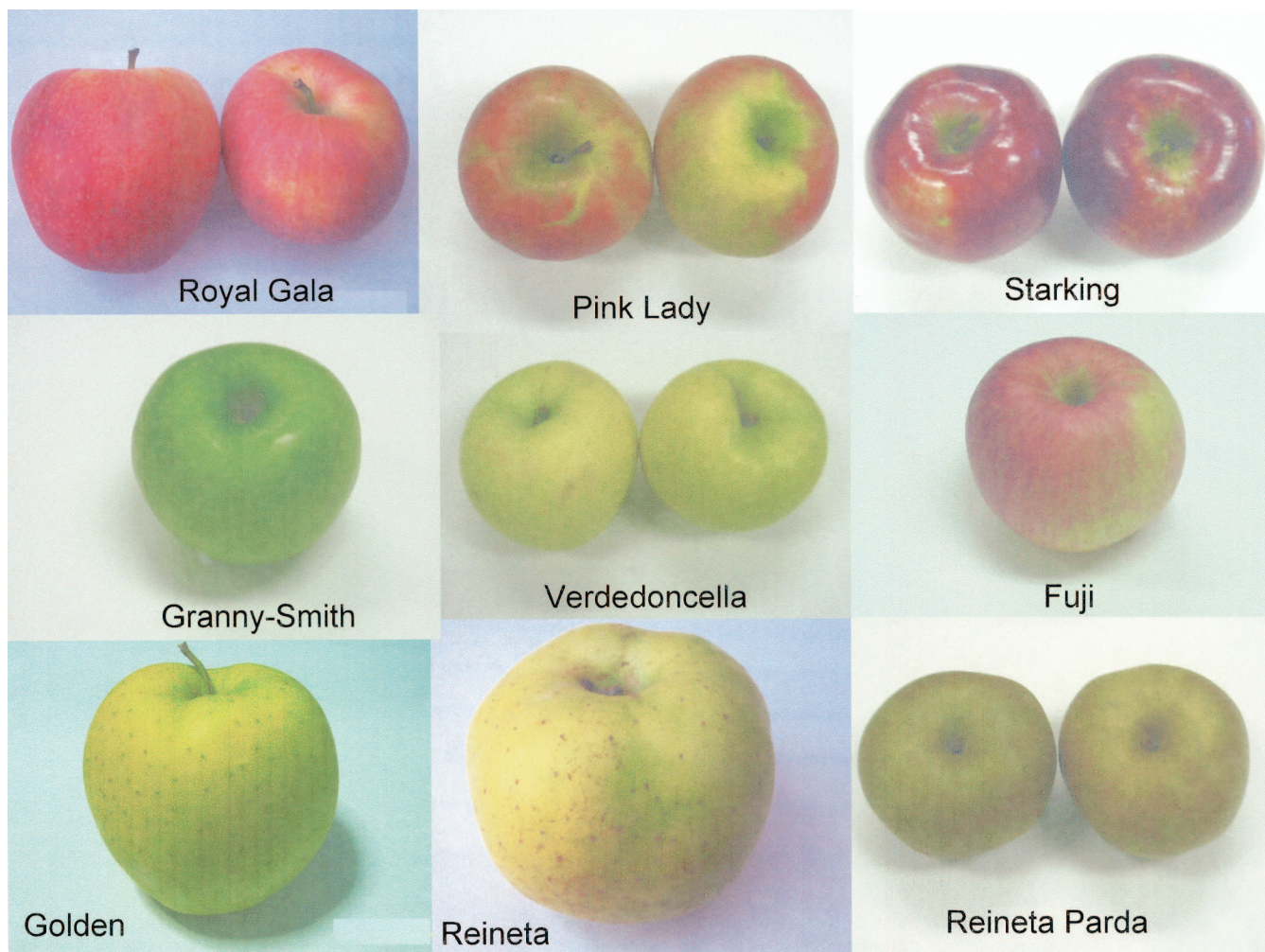


Figure 1. Ten different apple varieties were used in this study. Golden Perlum is not shown.

the apples were carefully peeled, and the peels from each variety were homogenized in phosphate-buffered saline (PBS)/polyvinylpyrrolidone buffer. After extraction for 4 hours under continuous magnetic stirring at 4°C, the extracts were centrifuged for 30 minutes at 10,000 rpm. The supernatants were collected and dialyzed overnight against bidistilled water, sterile filtered, frozen, and freeze-dried. The protein content was measured using the Lowry-Biuret method (BioRad Laboratories, Hercules, CA).

Patient Population

Twenty-two Spanish patients residing in Alicante (Mediterranean coast) who had a clinical diagnosis of IgE-mediated food allergy¹² (oral allergy syndrome after apple ingestion) were included in the study. Serum samples were collected, frozen, and stored at -20°. Eleven of these patients underwent skin prick testing (SPT) using extracts of Golden, Starking, and Reineta Parda at 5 mg/mL to determine differences in the *in vivo* allergenicity of these varieties.

Specific IgE

Specific IgE to apple, birch, and other fruits, such as peach, kiwi, and melon, was determined using the CAP system (Pharmacia Technologies, Uppsala, Sweden). Specific IgE binding to all peel extracts was determined in the 22 patients by enzyme-linked immunosorbent assay (ELISA). Briefly, 20 µg/mL of a freeze-dried extract 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 PBS (0.01M) and incubated (100 µL) for 2 hours in the wells. The plates were washed and incubated for 2 hours with α-human IgE (Ingenasa, Madrid, Spain) conjugated with peroxidase. After 5 washes, the reaction was developed for 30 minutes and then stopped with sulfuric acid (1N). Three individual serum samples from nonallergic patients were used as negative controls. Optical densities higher than 3 times the mean value of the negative control were considered positive.

Antigenic and Allergenic Profiles and Mal d 3 Content

The antigenic profile of the apple peel extracts was evaluated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Samples were run in a gel with 2.67% C, 15% T acrylamide/bisacrylamide as described by Laemmli.¹³ A 150- μ g freeze-dried sample from each variety was diluted in sample buffer, denatured at 100°C for 10 minutes, and centrifuged for 1 minute at 10,000 rpm. After electrophoresis, the gel was fixed for 45 minutes and silver stained (BioRad Laboratories). The allergenic profile of the Starking apple peel extract was determined using Western blot experiments and serum samples from 19 individuals. After electrophoresis, the gel was electrotransferred onto Immobilon membrane (Millipore, Bedford, MA). Membranes were incubated overnight with individual serum samples, diluted 1:5 in PBS Tween 0.1%. After washing, the membrane was incubated with peroxidase-conjugated monoclonal α -human IgE (Ingenasa). The Mal d 3 content of each variety was measured using scanning densitometry (Scanner Sharp JX-330) and was analyzed using the Image Master 1-D Elite v 4.00 (Pharmacia Biotech, Uppsala) in relationship to the total protein content of the extract.¹⁴

Statistical Analysis

The mean, SD, and median wheal sizes induced by the 3 extracts were calculated. The Shapiro-Wilks test was used to determinate normal distribution, and the Wilcoxon test was used to evaluate the differences between the wheal sizes induced by the 3 SPTs. Statistical analysis was also con-

ducted to determine IgE optical density values. Results were analyzed using the Kruskal-Wallis nonparametric test and the Bonferroni correction.

RESULTS

Patient Population

All 22 patients (17 women and 5 men; mean [SD] age, 24.6 [7.2] years) had clinical symptoms after apple ingestion. Seven patients (32%) tolerated the ingestion of apple pulp. The most common symptoms after apple ingestion were lip angioedema and oral allergy syndrome. Some patients also had symptoms after ingestion of peach ($n = 9$; 41%), nuts ($n = 6$; 27%), kiwi ($n = 3$; 14%), and melon ($n = 3$; 14%). Six patients (27%) did not show allergy to different aeroallergens, and 12 (55%) were allergic to pollen and 5 (23%) to mites.

Antigenic and Allergenic Profiles and Protein Contents

Findings from sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the 10 apple varieties are represented in Figure 2. Mal d 3 was present in all the extracts. A 34-kDa allergen is also clearly visualized in all extracts. The immunoblot experiments revealed that 13 (68%) of the 19 patients recognized Mal d 3. Other bands were recognized at molecular weights of 14, 18, and 66 kDa. Six patients (27%) recognized bands with higher molecular weights. The total protein and Mal d 3 contents of the varieties are shown in Figure 3. The Starking variety had the highest protein and Mal d 3 contents.

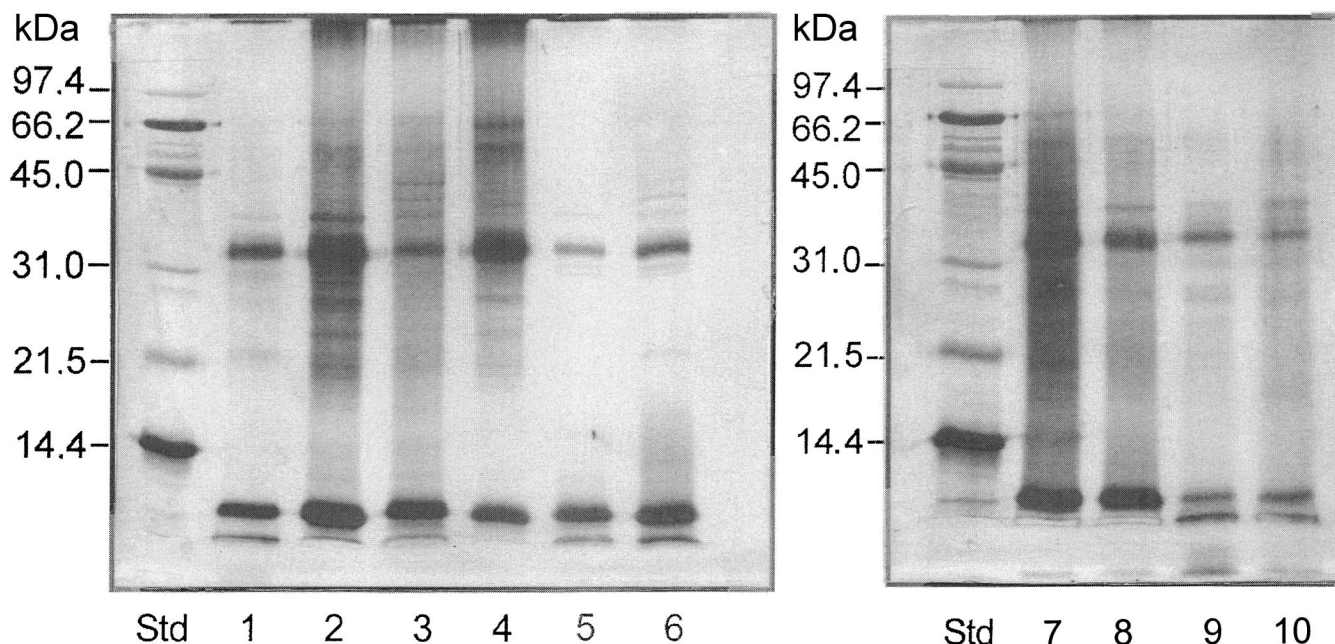


Figure 2. Findings from sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the 10 apple varieties used in this study. Lane 1, Royal Gala; lane 2, Pink Lady; lane 3, Starking; lane 4, Granny Smith; lane 5, Verdedoncella; lane 6, Fuji; lane 7, Golden; lane 8, Golden Perlim; lane 9, Reineta; and lane 10, Reineta Parda. Std indicates molecular weight standards.

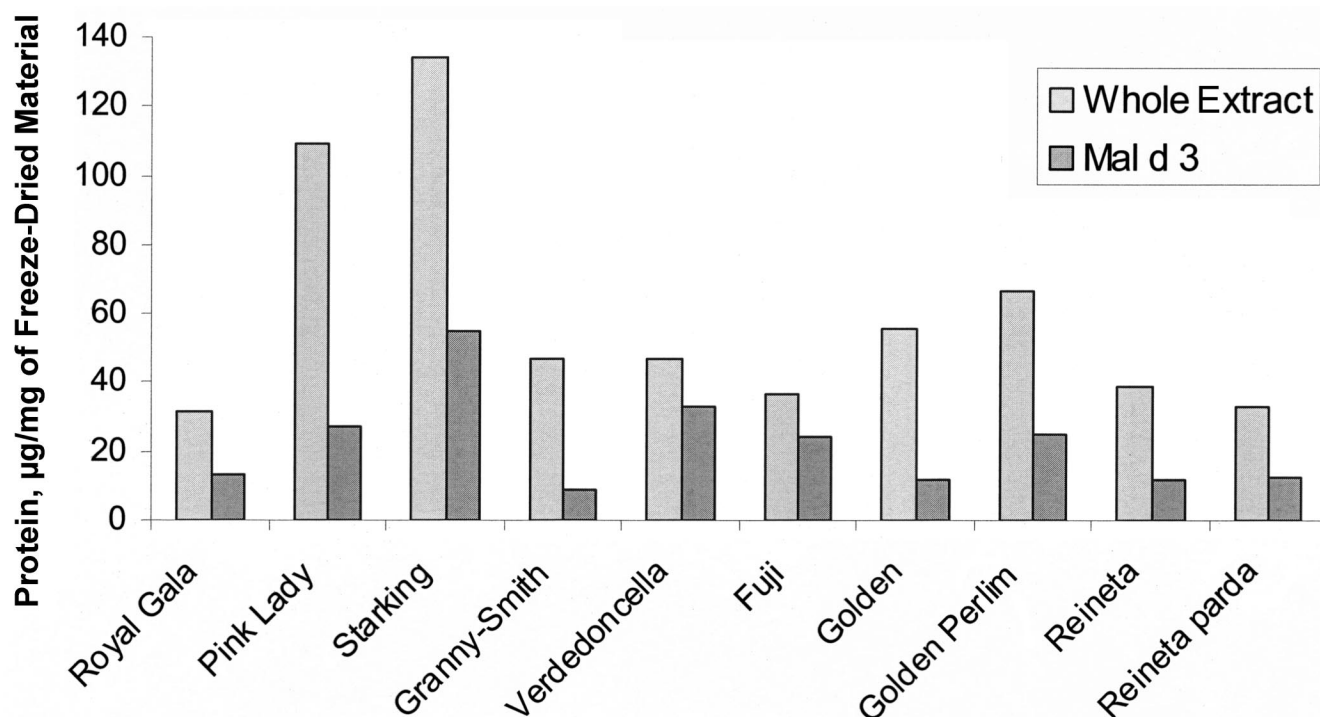


Figure 3. Total protein and Mal d 3 contents of the 10 apple varieties used in this study.

SPTs and Specific IgE Determinations

Skin prick tests were performed in 11 patients with peel extracts from Golden, Starking, and Reineta Parda apples at 5 mg/mL. All the patients had positive SPT results with the Starking variety; 2 patients had negative results with the Golden and 8 with the Reineta Parda varieties. Significant differences were found in the results (Wilcoxon signed rank test) between the Golden and Starking varieties ($P = .03$) and between the Reineta Parda and Starking varieties ($P < .01$) (Fig 4).

Seventeen patients (77%) had a positive specific IgE determination to apple using the Pharmacia CAP system; 3 (14%) were also positive to *Betula verrucosa*, 10 (45%) to peach, 3 (14%) to melon, and 1 (5%) to kiwi. Using direct ELISA experiments, 14 patients (64%) had detectable specific IgE to the Starking peel extract. A correlation coefficient of 0.741 was obtained between CAP and ELISA specific IgE determinations. The highest optical density values were obtained for the Starking variety; the Reineta Parda variety was the least recognized (Fig 5). Results were analyzed using the Kruskal-Wallis nonparametric test and the Bonferroni correction. Significant results were obtained between the Starking and Reineta Parda, Golden, Golden Perlim, and Royal Gala varieties ($P < .01$).

DISCUSSION

Previous studies^{15,16} have demonstrated the variability in the allergenic potency of commercial fruit extracts, including

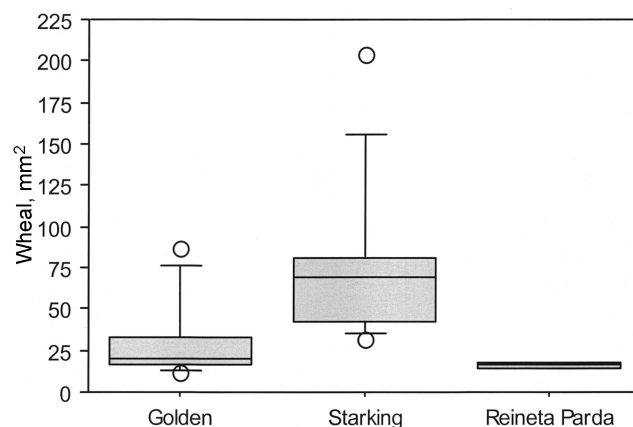


Figure 4. Box plot of the wheal sizes induced with peel extracts from Golden, Starking, and Reineta Parda apples at 5 mg/mL in 11 patients. Horizontal lines indicate the 10th, 25th, 50th, 75th, and 90th percentiles; white circles, values greater than the 90th percentile or less than the 10th percentile.

apples and peaches. For that reason, the objectives of this study were to compare the extracts of 10 different apple peel varieties and to describe the immunochemical characteristics of them. We decided to investigate apple peel extract 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.⁷ It is also

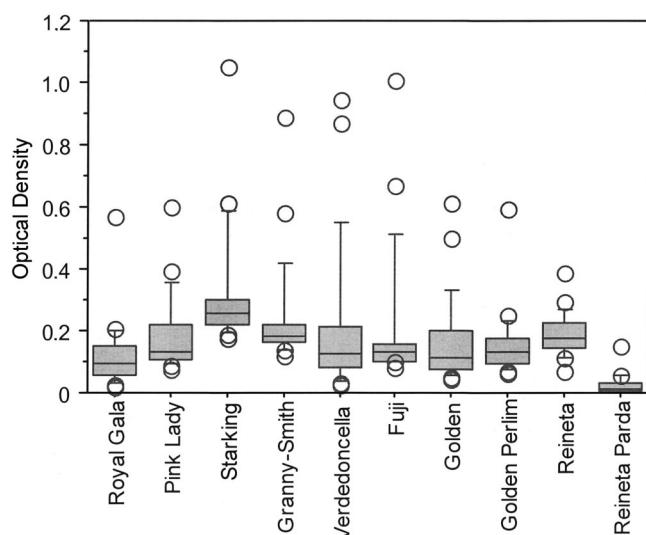


Figure 5. Box plot of the specific IgE binding results in 22 patients to the different analyzed apple varieties. Horizontal lines indicate the 10th, 25th, 50th, 75th, and 90th percentiles; white circles, values greater than the 90th percentile or less than the 10th percentile.

known that some apple-allergic patients experience symptoms after eating whole apples but may tolerate peeled fruits.¹⁷

A few studies^{18–21} have analyzed the allergenic composition of some of the most widely consumed apple varieties. All these studies confirmed that there are differences in the allergenic composition of the analyzed varieties. In these studies, the number of analyzed varieties was 16, 4, and 2, respectively. The patient population, the analyzed varieties, and the methods used were different, making it difficult to reach definitive conclusions.

We demonstrated the existence of differences between the antigenic profiles of the extracts. In all cases, 2 prominent bands of approximately 9 kDa, which could correspond to Mal d 3,²² and 32 kDa were visualized. Other proteins were also visible. The Verdedoncella and Fuji varieties showed fewer bands on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. However, the protein content varied significantly among apple varieties, from 133 $\mu\text{g}/\text{mg}$ of freeze-dried material in the Starking variety to 31 $\mu\text{g}/\text{mg}$ of freeze-dried material in the Royal Gala variety. In all cases, the apples were purchased at a similar ripening stage, and the extracts were prepared using previously described methods to prevent the loss of protein during the manufacturing process. Mal d 3 content was used as a marker to illustrate changes in the allergenic composition of the apple extracts because of its importance in Mediterranean countries.²³ Previous studies¹⁰ confirmed that most apple-allergic patients residing in Alicante have specific IgE to Mal d 3 and that cross-reactivity with birch pollen is not clinically relevant. The Mal d 3 content, measured using scanning densitometry, showed important differences among the 10 analyzed varieties, oscillat-

ing from 54 $\mu\text{g}/\text{mg}$ of freeze-dried extract in the Starking variety to 9 $\mu\text{g}/\text{mg}$ of freeze-dried extract in the Granny-Smith variety. The Starking variety had higher levels of total protein and Mal d 3 content. However, in the literature, the most commonly used variety is Golden, probably because it is the most common and the most consumed apple variety.²⁴ According to our results, this variety does not seem to be the ideal apple standard for use in the experiments, especially for diagnosis.

Twenty-two patients residing in the province of Alicante were enrolled in the present study. The patient population was selected based on a clinical history of apple sensitivity, confirmed using a prick-prick test (Golden variety) and a commercial apple peel extract. In all cases, patients experienced symptoms after apple ingestion. Forty-one percent of these patients also had positive SPT results with peach extracts, which could be explained by cross-reactivity between both fruits,²⁵ or cosensitization. In this study, 18 of 22 patients had a clinical history of sensitization, confirmed by SPT, to other Rosaceae fruits, including almonds, peaches, apricots, and cherries. Two patients did not show sensitization to other fruits, and 2 were sensitized only to melon. Ten patients were sensitized to different weeds, and 6 of them had positive SPT reactions to *Artemisia vulgaris* (mugwort). The LTP-mediated cross-reactivity between Rosaceae members and mugwort has been described previously.^{26,27}

Specific IgE binding to the 10 apple varieties was tested by direct ELISA in all patients. Important differences were found in specific IgE binding capacity to the extracts. The highest optical density values were obtained for the Starking variety. All the patients had a positive IgE determination to the Starking variety, whereas the results were all negative to Reineta Parda. It seems, therefore, that the Starking variety should be used to diagnose apple allergy in this population. Individual serum samples were used for the immunoblot experiments using the Starking variety; 68% of the patients recognized Mal d 3 (the 9-kDa band), suggesting that LTP is implicated in most apple-sensitized patients who are not sensitized to *Betula* pollen. Other allergens were also recognized.

To confirm the *in vitro* results, we selected 3 apple varieties (Starking, Golden, and Reineta Parda) based on their *in vitro* allergenic potency to SPT in 11 patients. The Starking variety induced the largest wheals, confirming the *in vitro* results. Statistical analysis revealed significant differences between the Starking and Reineta varieties and between the Starking and Golden varieties. No significant differences were found between the Golden and Reineta varieties. According to the *in vivo* and *in vitro* results, and to confirm the differences in the allergenicity of the apple varieties after ingestion, these results should be confirmed in oral provocation studies to elucidate the possibility that patients with apple allergy could tolerate the ingestion of some varieties. So far, no studies investigating this point have been published, to our knowledge.

We acknowledge that the present study represents a cross-sectional survey of the allergenicity of different apples purchased at a local supermarket. All the apples were bought on the same day and, therefore, represent the apple varieties commercialized during the summer of 2004. Based on our results, we suggest that the Starking variety was the most allergenic. Unless experiments are conducted with apples collected at different times of the year, from different cultivars around the world, and at different ripening stages, there is no answer to the question of whether the variability among varieties could represent variability within each variety. The lack of studies measuring protein content and allergenicity of the same apple variety in sequential years makes it difficult to draw a definitive conclusion on recommending consumption or avoidance of a certain apple variety to an apple allergic individual. Based on our results, we cannot recommend ingestion of a certain apple variety by apple-sensitive patients, especially because LTP and other allergens were present in all the extracts.

In conclusion, we demonstrated the existence of important differences in the antigenic and allergenic profiles and total protein and Mal d 3 levels in 10 different apple varieties purchased during the summer of 2004. These differences seem to be responsible for the different allergenicity observed. The identification of less allergenic, or hypoallergenic, apple varieties could be important in dietary recommendations, especially for patients with severe allergic symptoms. According to the in vitro and in vivo results, the Starking variety seems to be the most allergenic variety and the best candidate to use in diagnostic extracts; however, the analysis of more varieties and more oral challenges are needed to confirm these results.

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