

Allergenicity Assessment of Apple Cultivars: Hurdles in Quantifying Labile Fruit Allergens

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Key Words

Mal d 1 · Mal d 4 · Allergen quantification · Cultivar screening · Allergenicity

Abstract

Background: Assessment of allergenicity of foods is important for allergic consumers and regulators. Immunoassays to measure major food allergens are widely applied, often giving variable results. Using the major apple allergen Mal d 1 as a model, we aimed to establish at the molecular level why different immunoassays for assessing allergenicity of apple cultivars produce conflicting outcomes. **Methods:** Mal d 1 was measured in 53 cultivars from Italy and 35 from The Netherlands, using four different immunoassays. Purified Mal d 1 standards were molecularly characterized by size-exclusion chromatography (SEC) and mass spectrometry (MS). **Results:** Three immunoassays using an identical standard gave similar results. Minor differences in sample preparation already resulted in significant loss of allergenicity. The fourth assay, using a different Mal d 1 standard, gave 10- to 100-fold lower outcomes. By SEC, this standard was shown to be almost fully aggregated. This aggregation was accom-

panied by a decrease of the mass of the Mal d 1 molecule by ~1 kDa as analyzed by MS. The deviating immunoassay was shown to selectively recognize this aggregated form of Mal d 1, whereas the other three assays, including the one based on IgE antibody recognition, preferentially bound non-aggregated allergen. **Conclusions:** Variable and poorly controllable major allergen modification in both extracts and standards hamper accurate allergenicity assessments of fruits.

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Introduction

Apple allergy has been described most frequently as a cross-reactive phenomenon linked to birch pollinosis [1, 2]. Bet v 1 and Bet v 2 homologous proteins have indeed been identified as cross-reactive allergens in apple, Mal d 1 and Mal d 4, respectively [3–5]. More recently, a third birch pollen allergen, Bet v 6, was demonstrated to be involved in pollen-fruit cross-reactivity [6]. Allergy to fruits like apple can also occur independent from pollen sensitization [7]. A non-specific lipid transfer protein (LTP,

Mal d 3) was identified as major allergen for non-pollen-related allergy to apple [8, 9]. Recently, a 31-kDa allergen was also detected in apple [10] and later identified as a thaumatin-like protein (Mal d 2) [11].

In Central and Northern Europe, allergy to fruits and nuts is mostly associated with birch pollen and is almost exclusively mild and restricted to the oral cavity [12, 13]. In Southern Europe, sensitization to LTP plays an important role, resulting in far more severe symptoms [7, 14, 15].

Apple-allergic patients frequently report that they tolerate certain apples better than others. Various studies have demonstrated that the type of cultivar and the maturity of apples are indeed factors in their allergenicity [10, 16–18]. Certain plant proteins known as pathogenesis-related proteins (PR proteins) are upregulated in plants in response to wounding, environmental stress or infection by pathogens [19–21]. Mal d 1, Mal d 2 and Mal d 3 all belong to families of PR proteins (PR10, PR5 and PR14, respectively). For both apple consumers and growers it is worthwhile to assess the allergenicity of different apple cultivars. Apple-allergic patients with exclusively mild oral symptoms (mostly Mal d 1-reactive patients) might want to continue eating low allergenic apples because of their value for a healthy diet or simply because they like them. For Mal d 3-reactive patients, the increased risk of severe reactions probably justifies the advice to also avoid apples with decreased allergenicity, even when severe reactions have not (yet) occurred.

How reliably can the allergenicity of apples be assessed? Recently, we reported the use of the prick-to-prick method with fresh apples as a reproducible way to measure allergenicity [18]. Of course, this is not a feasible approach for high-throughput screening of large panels of cultivars. In that case, *in vitro* immunoassays are more suitable.

Recently, in a large European multicenter study, we aimed at assessing the allergenicity of 88 apple cultivars using various immunoassays. 53 cultivars were grown in Ferrara (Italy) and an additional panel of 35 apple cultivars was grown in Wageningen (The Netherlands). The Mal d 1 levels of 39 cultivars grown in Italy obtained with a single enzyme-linked immunosorbent assay (ELISA) were reported previously [22] and these levels were in close agreement with those reported earlier by other investigators. In that paper, conflicting data obtained with the other assays and the Dutch cultivars were not reported. The subject of this paper is to unravel the basis for the observed differences.

In this study, we report the full set of data for all cultivars using multiple immunoassays for the detection of

Mal d 1. For reasons of comparison, we included assays for the measurement of the stable allergen, Mal d 3 and for a second labile allergen Mal d 4. The aim of this study was to investigate what caused the inter-assay and inter-laboratory discrepancies. Such knowledge is essential to provide consumers and breeders with reliable information that can be used to rank apple cultivars with respect to allergenicity.

Materials and Methods

Apple Extraction

Apples of 35 different cultivars from Wageningen (The Netherlands) were harvested at a stage of ripeness used routinely by growers and stored for 3 months at 2°C before extraction. Five apples per cultivar were peeled (5 mm) with a paring machine and frozen in liquid nitrogen. This frozen material was extracted essentially according to Björkstén et al. [23]. In short, 5-mm-thick peels were mixed in a blender with extraction buffer (10 mM K₂HPO₄, 10 mM KH₂PO₄, 0.27 mM EDTA, 13.3 mM DIECA, 2% PVPP, pH 7) at a ratio of 1:2 w/v, in the presence of a protease inhibitor cocktail (Roche, Cat. No. 1 836 145/1 697 498, Germany) and stirred at +4°C for 30 min. The mixture was centrifuged at 10,000 g for 30 min at 4°C. Subsequently, the supernatant was filtered over a black ribbon filter (Schleicher & Schüll, Dassel, Germany), using a Büchner funnel (vacuum) and subsequently frozen in 2-ml aliquots at –30°C.

Apple cultivars (n = 53) originating from Ferrara (Italy) were extracted following an identical protocol that in retrospect appeared to contain two deviations. Apples were powdered in liquid nitrogen before mixing with extraction buffer and extracts were filtered through Miracloth (Calbiochem Merck KGaA, Darmstadt, Germany), aliquotted and stored at –80°C. Frozen aliquots of all extracts were exchanged between three centers performing allergen measurements. Shipment was on dry ice and aliquots were immediately stored at ≤–30°C.

Isolation and Purification of nMal d 1 and rMal d 4

Mal d 1 was affinity-purified from apple extract of cultivar Golden Delicious using monoclonal antibody (mAb) 5H8 as described elsewhere [24]. One batch of Mal d 1 was purified from an extract produced with an identical protocol as used for the Dutch cultivars. This Mal d 1 was used as standard in ELISA-Bet v 1, competitive radioimmunoassay (cRIA) and RAST inhibition (RI) but not in ELISA-Mal d 1. For the latter assay, Mal d 1 was affinity-purified from an extract of Golden Delicious obtained using an extraction protocol originally developed for isolation of profilins [25]. In brief, fresh apples (with peel, without core) were mixed in a blender with PHEM-Tx buffer (66 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM MgCl₂, 5 mM glucose) containing 10 µg/ml leupeptin, 78 µg/ml benzamidin, 47 µg/ml aprotinin, 0.5 mM PMSF and 0.75% Triton X-100, at a ratio of 1:2 w/v and extracted by stirring at +4°C for 2 h. The most important difference with the first protocol is the absence of PVPP. Therefore, the two different Mal d 1 preparations will be referred to as PVPP^{plus}-Mal d 1 and PVPP^{minus}-Mal d 1, respectively. Recombinant Mal d 4 was expressed in *Escherichia coli* and affinity-purified using poly(L-proline) Sepharose as described by Y. Ma et al. [26].

Antibodies

Production of Mal d 1-specific mAb 4C3C10 as well as polyclonal antibodies (pAb) was described previously by Marzban et al. [22]. Here it is relevant to mention that this mAb was raised using PVPP^{minus} as immunogen. To produce the pAbs, recombinant Mal d 1 (Biomay, Vienna, Austria) was used.

mAb 5H8 was raised with natural Bet v 1 as described by Akkerdaas et al. [24]. For pAbs to Bet v 1, New Zealand White rabbits were immunized with 50 µg natural Bet v 1 in Montanide ISA-50 (Seppic, Paris, France). Boosters were given at 4-week intervals, and plasma was collected at days 7, 9 and 11 after boosters 4, 5 and 6. Plasma was recalcified and antibody titers were measured by radioallergosorbent test (RAST) with Sepharose-coupled Bet v 1. On the basis of this analysis, sera were pooled and stored frozen at -30°C. Cross-reactivity was assessed by RAST with Sepharose-coupled food extracts. This resulted in two pAbs (123766 and 123767). For application in ELISA, IgG was purified using caprylic acid [27]. pAbs against grass pollen profilin (Lol p 12) were produced and characterized following the same protocol [28]. pAb and mAbs against natural Mal d 3 were produced as described previously [29].

Assays

Enzyme-Linked Immunosorbent Assay

A sandwich ELISA was developed for the measurement of nMal d 1 based on both polyclonal rabbit anti-Bet v 1 antibodies (123766 for coating, 123767 for detection). Microtiter plates (Maxisorp, Nunc, Denmark) were coated overnight at 4°C with 100 µl rabbit anti-Bet v 1 at 2 µg/ml in coating buffer (50 mM NaHCO₃, pH 9.6). After blocking with phosphate-buffered saline (PBS)/0.1% Tween-20/1% bovine serum albumin (PBS-AT), wells were incubated with (serial) dilutions of apple extracts in duplicate. Detection was performed with 2 µg/ml rabbit anti-Bet v 1 in conjunction with peroxidase-conjugated goat anti-rabbit IgG antibodies (Dako, dilution 1:1,000). All dilutions of samples, references and reagents were made in PBS-AT. All incubations were carried out for 1 h at room temperature except for the enzyme conjugate which was incubated for 30 min at 37°C. Washing was done with PBS/0.05% Tween-20 (PBS-T). Finally, the plates were incubated in the dark at room temperature for 20 min with substrate (1 mM ABTS/0.03% H₂O₂ in 0.1 M citric acid/0.1 M sodium citrate, pH 4.0). Addition of 2% oxalic acid stopped the reaction. Mal d 1 concentrations were calculated by parallel-line analysis using PVPP^{plus}-Mal d 1 as calibration curve. This ELISA is in the rest of the article referred to as Bet v 1-ELISA.

The second sandwich ELISA has been described previously [22]. It is based on mAb 4C3C10 raised against PVPP^{minus}-Mal d 1 and pAb anti-recombinant Mal d 1. In this assay, PVPP^{minus} was used as calibration curve.

ELISA for Mal d 3 has been described elsewhere. It is a double mAb-based assay [29].

Competitive Radioimmunoassay

A cRIA was performed essentially as described earlier [28], using mAb 5H8. This mAb was originally raised against Bet v 1 and has a well-established cross-reactivity to various PR10 allergens in fruits and nuts, including Mal d 1 [24]. In short, 50 µl mAb 5H8 (diluted 1:5,800 in PBS-AT) was incubated with (serial) dilutions of apple extract in 50 µl PBS-AT. After 2 h, 0.5 mg Sepharose-coupled goat anti-mouse antibodies and ¹²⁵I-radiolabeled

nBet v 1 (20,000 cpm) were added in a final volume of 900 µl PBS-AT. After overnight incubation and washing, bound radioactivity was measured in a gamma counter.

A cRIA for measuring Mal d 3 using pAb against Mal d 3 was described previously [29]. Finally, for measuring Mal d 4, 50 µl rabbit anti-Lol p 12 (1:8,000 in PBS-AT) was used in combination with Protein A-Sepharose (0.5 mg) and ¹²⁵I-radiolabeled rMal d 4 (20,000 cpm) in a final volume of 900 µl PBS-AT. Mal d 1, Mal d 3 and Mal d 4 concentrations were calculated by parallel-line analysis using a standard curve of purified PVPP⁺-Mal d 1, Mal d 3 or rMal d 4, respectively.

For comparing the immunoreactivity of mAb 5H8 and 4C3C10, a non-cRIA was performed using different mAb dilutions. In addition to radiolabeled Bet v 1, radiolabeled PVPP^{plus} and PVPP^{minus} were used. A subgroup of cultivars was assessed for Mal d 1 content in a cRIA using mAb 4C3C10 (1:50 diluted in PBS-AT) and radiolabeled PVPP^{minus}-Mal d 1.

Indirect RAST

For measuring Mal d 4, an indirect RAST was used as described earlier [30]. In short, 2.5 mg poly(L-proline) (MW 42,700, Sigma) was coupled to 100 mg of CNBr-activated Sepharose 4B. Apple extract (50 µl) was preincubated for 4 h with 0.5 mg Sepharose in a final volume of 300 µl PBS-AT. The Sepharose was washed 5 times with PBS-T prior to addition of 50 µl in-house reference serum with IgE antibodies specific for profilins. Subsequent steps were identical to those described for the RAST inhibition. Results were expressed as percent binding of radiolabeled anti-IgE. Calculation of Mal d 4 content was performed by parallel-line analysis using a standard curve of rMal d 4.

RAST Inhibition

Mal d 1 RI was based on the RAST procedure described previously [31]. Affinity-purified PVPP^{plus}-Mal d 1 (100 µg) was coupled to 100 mg of CNBr-activated Sepharose 4B (Amersham Biosciences, Uppsala, Sweden). A mixture of three in-house reference sera with IgE antibodies directed to Mal d 1 was preincubated for 2 h with (serial) dilutions (in 50 µl PBS-AT) of apple extract prior to addition of Sepharose-coupled Mal d 1. Subsequent steps were identical to those described for the RAST [31]. Calculation of Mal d 1 content was performed by parallel-line analysis using a standard curve of PVPP^{plus}-nMal d 1.

Radiolabeling

nMal d 1, nMal d 3 and rMal d 4 were radiolabeled as described before. Free iodine and labeled allergens were separated by size-exclusion chromatography (SEC) [32].

Mass Spectrometry

PVPP^{plus}- and PVPP^{minus}-Mal d 1 samples were analyzed by HPLC-electrospray mass spectrometry (ES-MS) as described by Moreno et al. [33].

PVPP^{plus}-Mal d 1 was subjected to HPLC-electrospray ionization quadrupole-time-of-flight mass spectrometry (ESI Q-TOF MS). Samples were dissolved in 0.1% aqueous formic acid at a concentration of approximately 100 fmol/µl. For the denaturing experiments, samples were heated at 82°C for 5 and 10 min, respectively. ESI-MS spectra were recorded on a Micromass Global Ultima Q-ToF instrument (Waters), coupled with a Waters capillary HPLC via a stream select valve. 500 fmol of sample were loaded

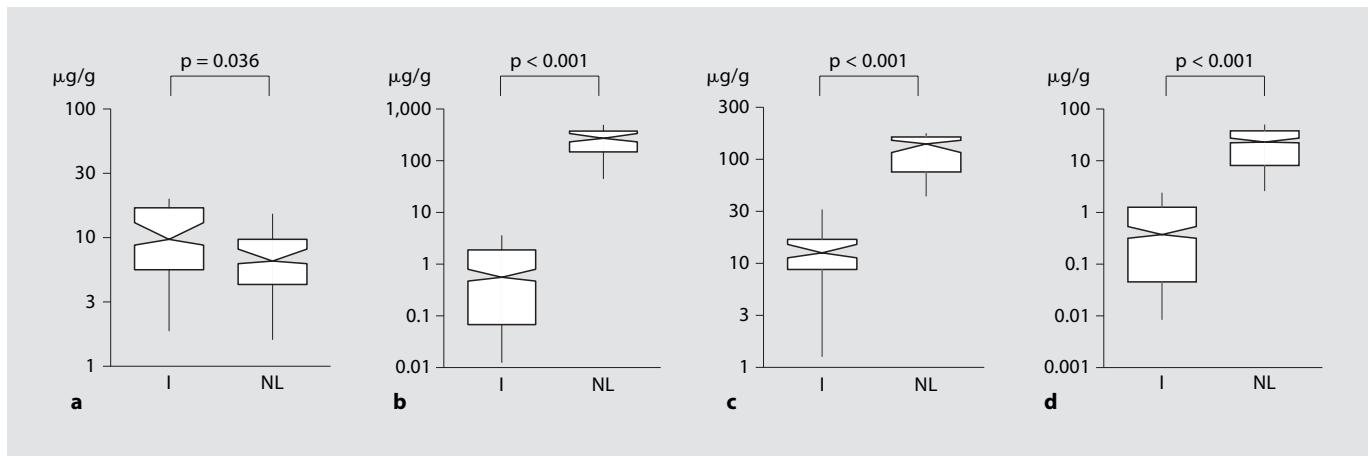


Fig. 1. Mal d 1 levels ($\mu\text{g/g}$ apple) determined by different assays: Mal d 1-ELISA (a), Bet v 1-ELISA (b), and RAST inhibition(c, d). The median, the 25- and 75-percentiles and the 10–90 percentiles are indicated.

on the trapping column (PepMap300 C18, 5 μm , 300 $\text{\AA}$, 300 $\mu\text{m} \times 1 \text{ mm}$ (LCPackings) and salts and other contaminants not binding to the column material were removed by washing the column at 20 $\mu\text{l/min}$ for 5 min. After completion of the wash step, the stream select valve was switched to the on-line position and proteins were eluted from the trapping column to the mass spectrometer by a stepwise increase of the acetonitrile concentration (15, 30, 45 and 60% acetonitrile in 0.1% formic acid). Each step lasted 2 min. During elution, mass spectra were continuously recorded in a mass/charge range from 400 to 1,900. Spectra containing multiply charged protein signals were combined and deconvoluted using the MaxEnt1 software supplied with the instrument.

Statistical Analysis

Results from different assays were correlated using Spearman rank correlation. Within one assay, median Mal d 1 levels and 10-, 25-, 75-, and 90-percentiles were calculated separately for I cvs and NL cvs, respectively. Differences between medians were assessed by Mann-Whitney U-test. With a p value < 0.05 a difference was regarded significant.

Results

Mal d 1 Measurements

Extracts of 53 apple cultivars (cvs) grown in Italy (I) and 35 grown in The Netherlands (NL) were analyzed for Mal d 1 content using four different immunoassays. Five cvs (Golden Delicious, Granny Smith, Braeburn, Fuji and Jonagold) were present in both groups. When analyzed by Mal d 1-ELISA using PVPP^{minus}-Mal d 1 as standard, both groups of cvs demonstrated a similar range of Mal d 1 levels (fig. 1a). Median Mal d 1 levels

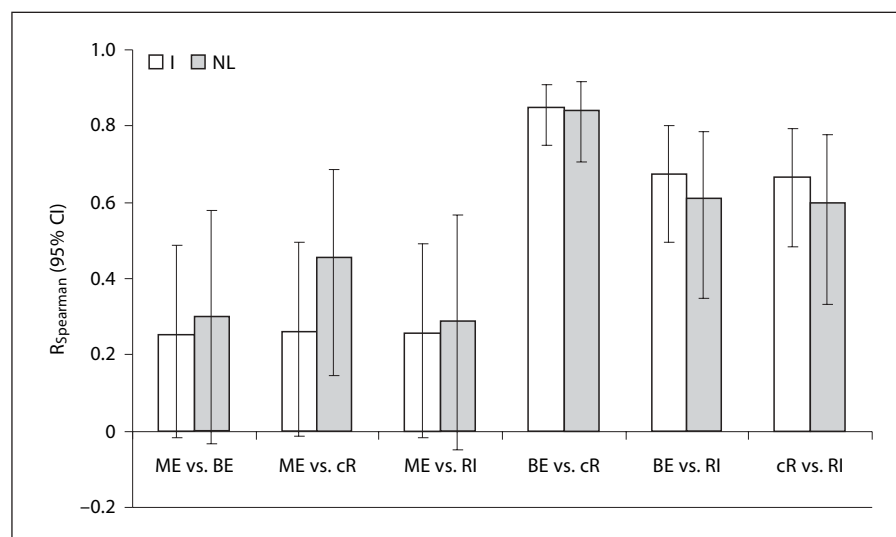
Table 1. Differences in Mal d 1 levels ($\mu\text{g/g}$ apple) as determined in the Bet v 1-ELISA for the cultivars present in both the Italian group and Dutch group

	Mal d 1, $\mu\text{g/g}$ ($\pm$ SD)	
	Italian	Dutch
Golden Delicious	14.10 $\pm$ 1.50	135.17 $\pm$ 12.53
Granny Smith	5.95 $\pm$ 0.25	18.17 $\pm$ 3.51
Braeburn	9.45 $\pm$ 0.3	271.20 $\pm$ 119.74
Fuji	32.84 $\pm$ 4.86	455.01 $\pm$ 7.17
Jonagold	12.98 $\pm$ 0.66	38.82 $\pm$ 9.09

did however differ significantly: 9.7 vs. 6.6 $\mu\text{g/g}$ ($p = 0.036$). Median Mal d 1 levels found in the Bet v 1-ELISA, cRIA and RI were 10.3, 12.4 and 0.4 $\mu\text{g/g}$, respectively for the I-cvs and 271.2, 134.1 and 23.2 $\mu\text{g/g}$, respectively, for the NL-cvs (fig. 1b–d). The differences between both groups of cvs in each of the assays (approximately 25-, 10- and 60-fold, respectively) were highly significant ($p < 0.0001$). Also for the cvs present in both panels, a significant difference was observed (shown in table 1 for the Bet v 1-ELISA).

No significant correlation between the Mal d 1-ELISA and the other three assays, with the exception of the cRIA for the NL-cvs, was shown by Spearman rank correlation (fig. 2). In contrast, the three assays demonstrated a good correlation amongst themselves with the strongest correlation found between the Bet v 1-ELISA and the cRIA ($R_{\text{Spearman}} > 0.84$).

Fig. 2. Spearman correlation coefficients indicating the difference in significance between the Mal d 1- and Bet v 1-based immunoassays in the I and NL cvs. ME = Mal d 1-ELISA; BE = Bet v 1-ELISA; cR = competitive RIA; RI = RAST inhibition.



In order to assess whether the unexpected difference between both groups of cvs observed in 3/4 assays was related to the minor differences in extraction protocols that had occurred, apples from 9 cvs (4 from I and 5 from NL) of the next year's harvest were extracted again using the extraction protocol originally used for the NL-cvs. Mal d 1 levels were determined in the three assays that had demonstrated differences (fig. 3). The NL-cvs of both harvests showed Mal d 1 levels of the same order of magnitude staying within a range of 2-fold lower to 4.5-fold higher. In contrast, the I-cvs gave significantly higher Mal d 1 titers with differences up to >1,000-fold. The absolute Mal d 1 values for the I-cvs of the second harvest were now in the same range as those measured for the NL-cvs. These data support the conclusion that the extraction of the I-cvs from the first harvest resulted in a loss of the overall immunoreactivity of Mal d 1.

Measurement of Mal d 3 and Mal d 4

To further investigate whether differences between I-cvs and NL-cvs were indeed related to a difference in the level of conservation of labile allergens during extraction, two additional apple allergens were measured in all cvs, Mal d 3 being an extremely stable allergen less likely to be affected by minor extraction and storage differences, and Mal d 4 being a relatively labile allergen (fig. 4). Although for both allergens, the median levels were very similar in the two groups of cvs, the quantities of Mal d 4 were moderately (2-fold) but significantly ($p < 0.01$) higher for the NL-cvs. For the stable allergen, Mal d 3 median levels were not significantly different.

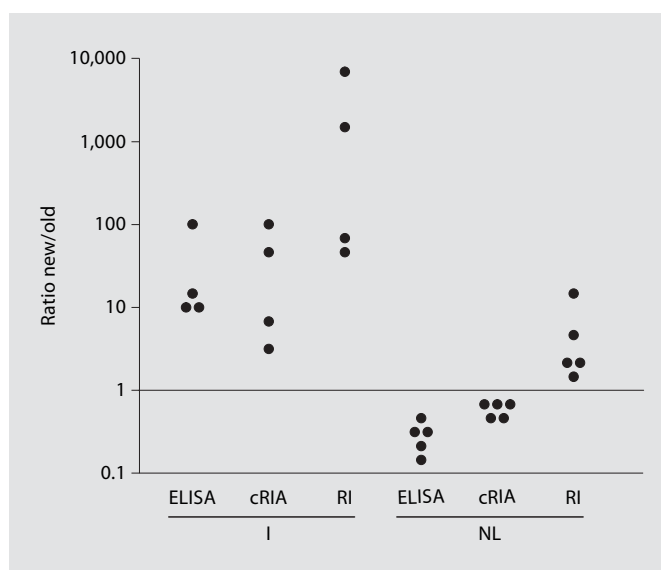


Fig. 3. The ratio between the Mal d 1 levels as determined in a selected group of cvs from harvest 2004 compared to the levels in the same group as determined in the harvest from 2003. Assays used as indicated on the axes. The extraction of the cvs from 2004 were all done in Amsterdam.

Differences in Specificity of Mal d 1-Reactive Antibodies and Assays

As outlined above, the substantial differences in Mal d 1 levels between I-cvs and NL-cvs observed within single assays can most likely largely be attributed to extraction- and/or storage-related factors. However, this cannot

Fig. 4. Mal d 3 (a) and Mal d 4 (b) levels ($\mu\text{g/g}$ apple) determined in all cvs harvested in 2003. The median, the 25- and 75-percentiles and the 10–90 percentiles are indicated.

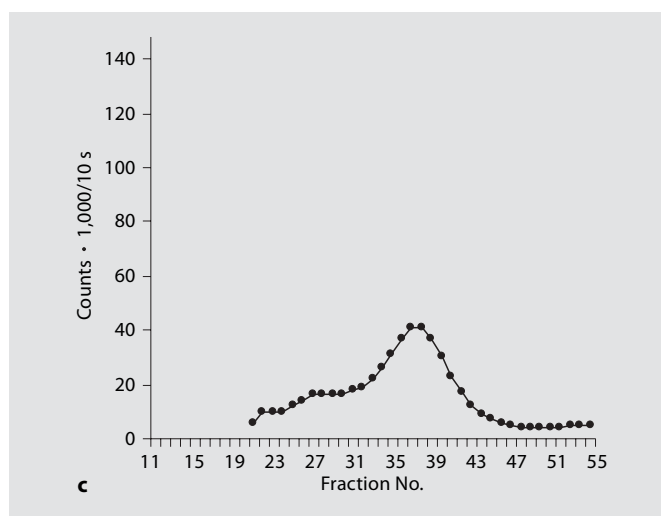
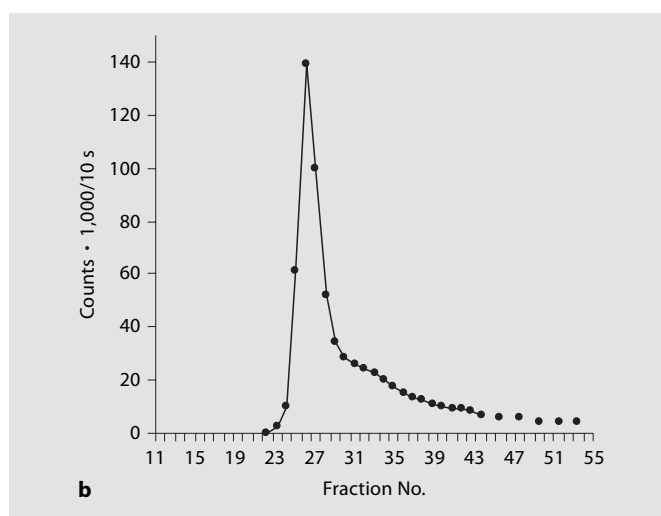
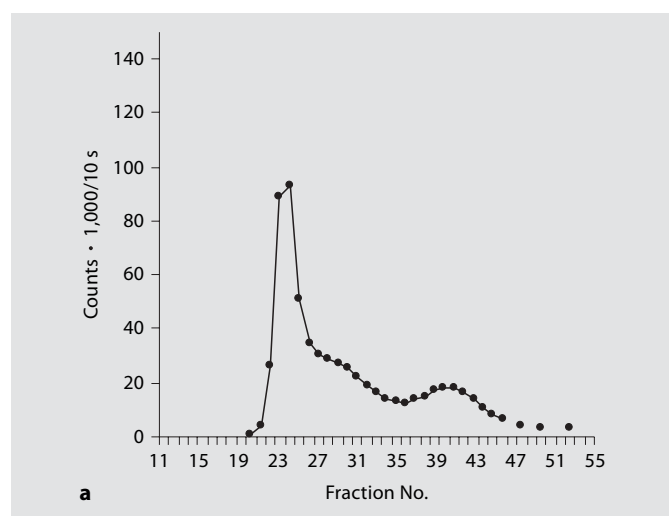
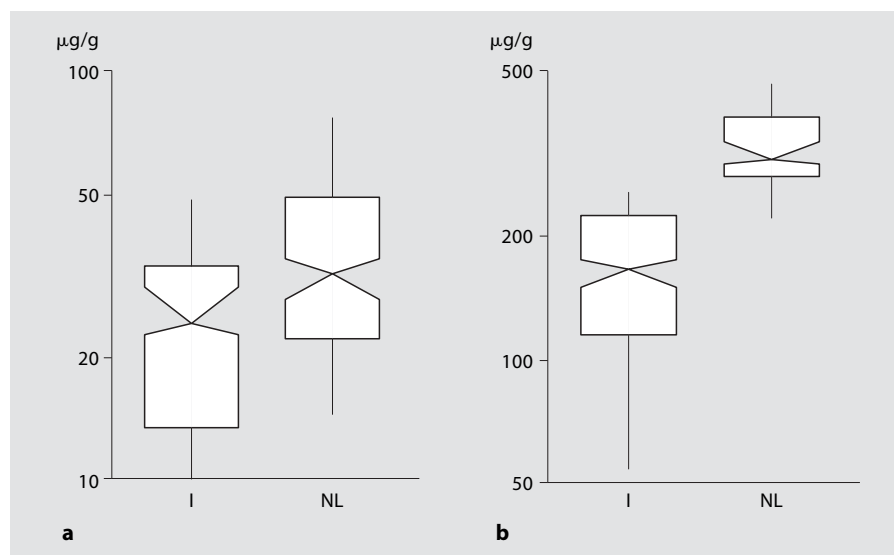


Fig. 5. Size-exclusion chromatography of ^{125}I -radiolabeled PVPP^{plus}-Mal d 1 (a), PVPP^{minus}-Mal d 1 (b), or Bet v 1 (c).

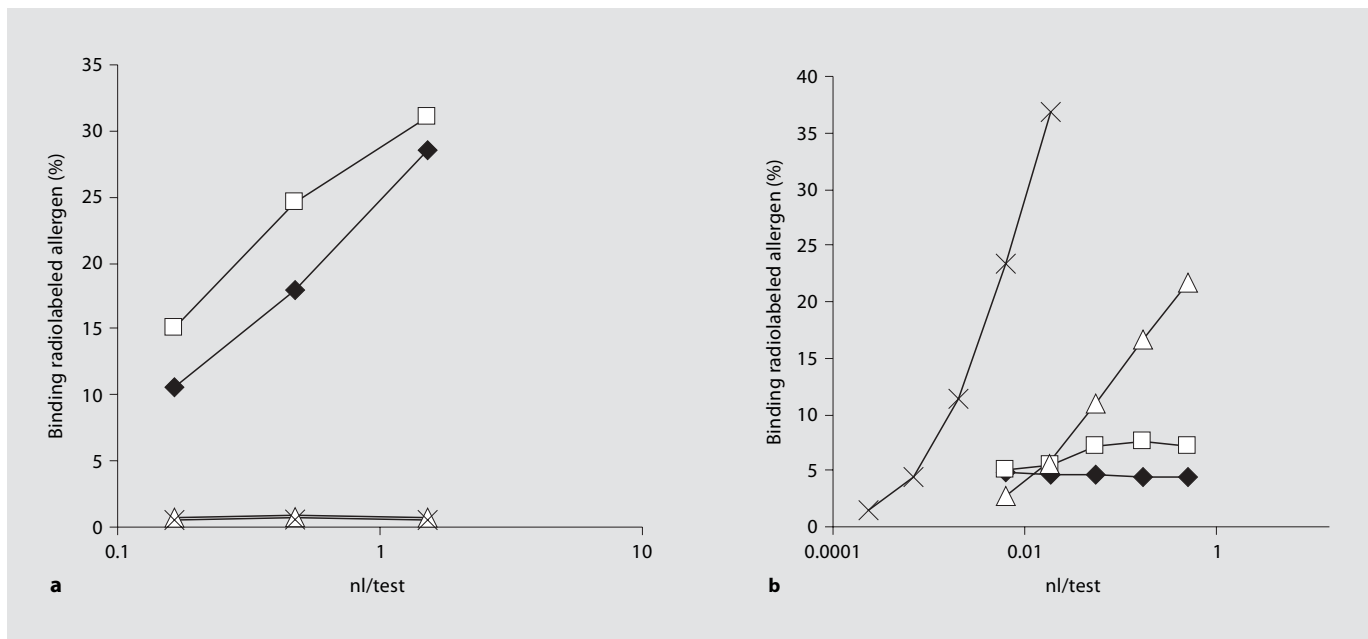


Fig. 6. cRIA with mAb 4C3C10 (a) and mAb 5H8 (b) and the different labeled allergens. Bet v 1 (×), PVPP^{minus}-Mal d 1 (◆), PVPP^{plus}-Mal d 1 (peak A, □) and PVPP^{plus}-Mal d 1 (peak B, △).

explain why NL-cvs were high in three assays (cRIA, Bet v 1-ELISA and RI) and low in the fourth (Mal d 1-ELISA). Since the latter assay used PVPP^{minus}-Mal d 1 as standard and the former three assays PVPP^{plus}-Mal d 1, both standards were subjected to further analysis. Radiolabeled PVPP^{plus}- and PVPP^{minus}-Mal d 1 were each separated by SEC (fig. 5). In both preparations, the major peak (fig. 5a) was in the void volume of the column, suggesting aggregation of the protein. However, PVPP^{plus}-Mal d 1 contained a second minor peak (fig. 5b) at the expected elution volume for non-aggregated Mal d 1 that was absent in PVPP^{minus}-Mal d 1. For comparison, radiolabeled nBet v 1, having a similar size as non-aggregated Mal d 1, was also included in the analysis. As expected, it eluted at the same volume as non-aggregated Mal d 1. Subsequently, immunoreactivity of mAb 5H8 and mAb 4C3C10 was tested towards radiolabeled aggregated and non-aggregated Mal d 1 and Bet v 1 (fig. 6). While 5H8 (used in the cRIA) primarily recognized Bet v 1 and non-aggregated Mal d 1, 4C3C10 (used in the Mal d 1-ELISA) recognized the aggregated form of Mal d 1 exclusively. It is likely that these differences in specificity play a role in the different levels of Mal d 1 obtained with assays based on both mAbs.

To investigate whether the low Mal d 1 levels detected in the Mal d 1-ELISA compared to the other three assays

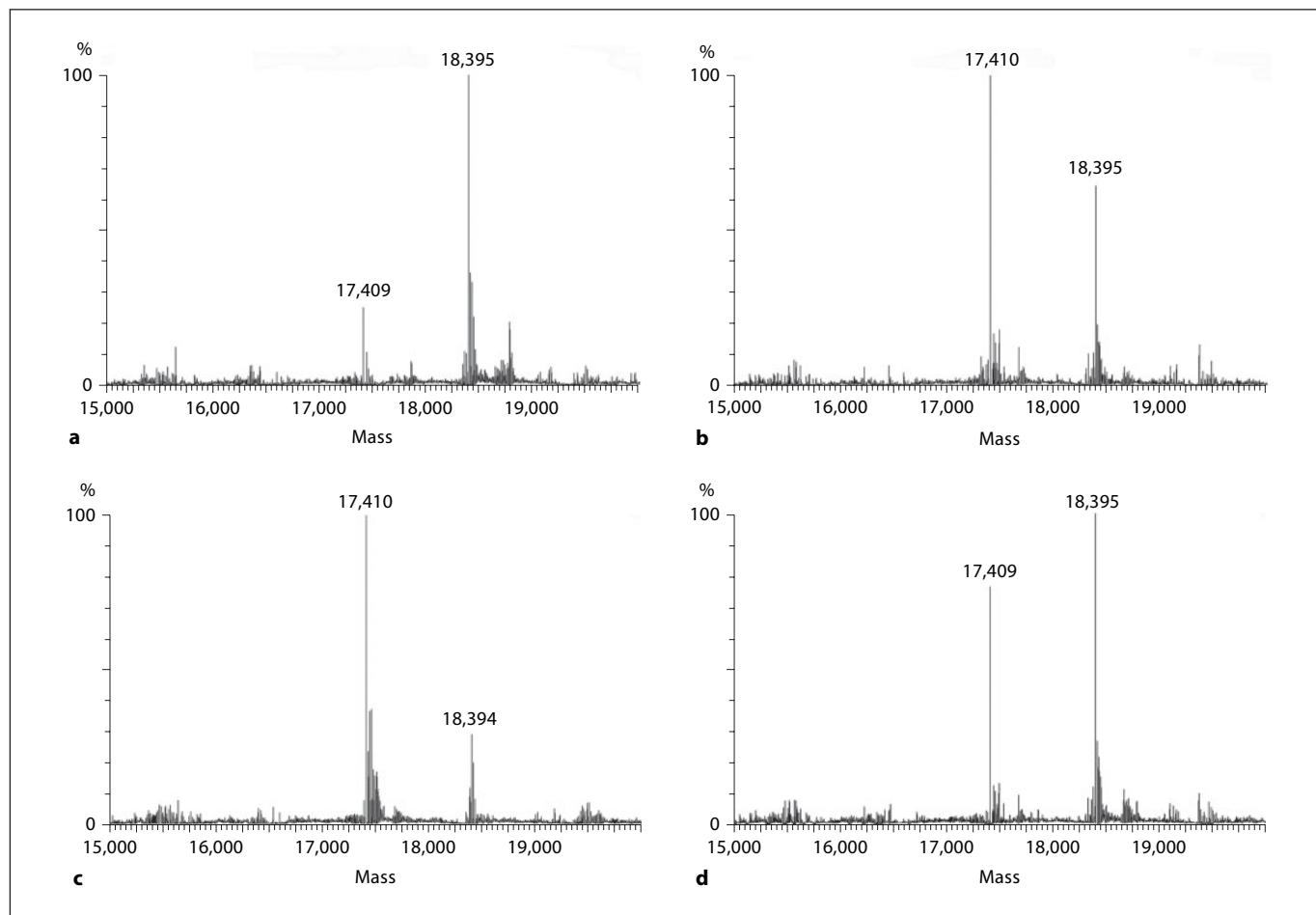
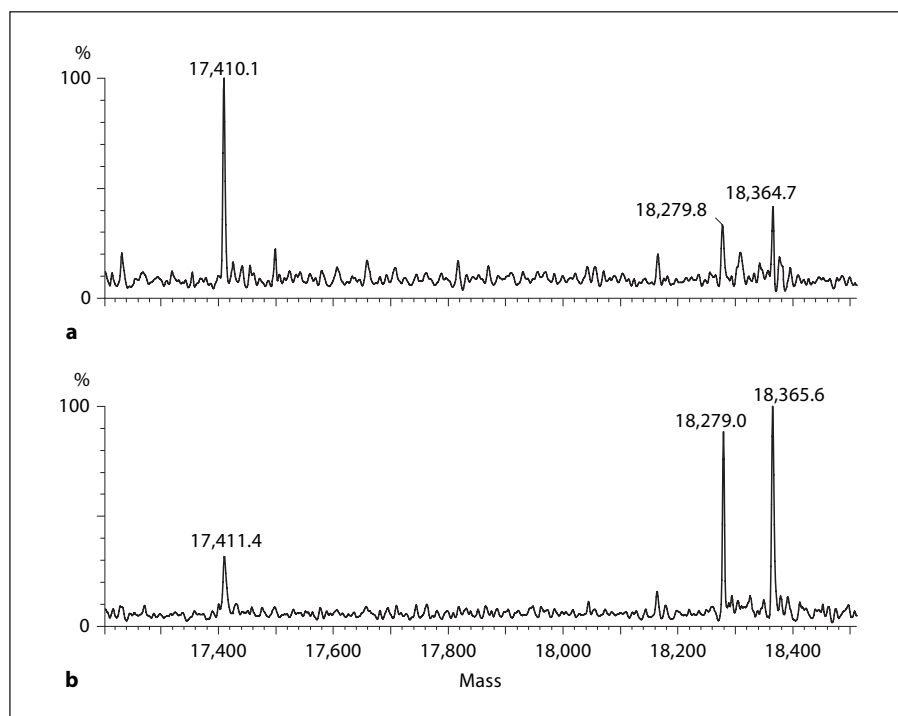
was indeed caused by the specificity of mAb 4C3C10, a cRIA was performed with mAb 4C3C10 instead of 5H8 and radiolabeled aggregated Mal d 1 instead of Bet v 1. Samples of 9 cvs from two subsequent harvests were used plus NL-cv Golden Delicious from the first harvest. As observed previously in the Mal d 1-ELISA, all values obtained with this RIA were in the 1- to 10- μ g/g range, in contrast to the 10- to 100 μ g/g range found with mAb 5H8 (not shown).

Mass Spectrometric Analysis of Mal d 1 Standards

To further compare PVPP^{plus}- and PVPP^{minus}-Mal d 1, both preparations were analyzed using ESI-MS. Both gave three peaks at M_r 17,410.0, M_r 18,282.5 and M_r 18,368.5 Da, respectively (fig. 7). The mass of the lowest peak corresponded closely to the theoretical mass of known Mal d 1 isoforms [17]. PVPP^{plus}-Mal d 1 was dominated by both higher molecular mass peaks. In PVPP^{minus}-Mal d 1 the share of the low molecular mass peak was significantly increased. To investigate whether storage-related factors could induce a shift from high to low molecular mass, PVPP^{plus}-Mal d 1 was subjected to heat treatment and freeze-thawing. Both procedures induced a significant increase of the low molecular mass peak at the cost of the high molecular mass as analyzed by ESI Q-TOF MS (fig. 8).

Fig. 7. ES-MS analysis of PVPP^{minus}-Mal d 1 (a) and PVPP^{plus}-Mal d 1 (b).

Fig. 8. ESQ-MS analysis of PVPP^{plus}-Mal d 1: untreated (a), after 5 min heating at 82°C (b), after 15 min heating at 82°C (c), and after a freeze-thaw cycle (d).



Discussion

It has been known for 25 years that extraction of fruits like apple requires special precautions in order to preserve IgE reactivity [16, 23, 34–37]. Oxidation by polyphenol oxidases in the presence of phenolic compounds that are released upon disruption of plant cells, modify proteins resulting in loss of allergenicity. Peroxidases and proteases can also contribute to this process [16, 38]. Mal d 1 is known as a labile protein that is easily affected by these endogenous enzymes. Its labile characteristics are emphasized by its sensitivity to heating [39, 40] and pepsin digestion [41, 42]. In contrast, Mal d 3 is known as extremely stable, resisting thermal denaturation [43–45] and pepsin digestion [42]. To prevent the detrimental effect of polyphenol oxidases and other enzymes, Björkstén et al. [23] developed a protocol that aimed at preserving allergenicity by capturing phenolic compounds by addition of PVPP and inhibiting enzymatic activity by the presence of a chelator of bivalent ions (DIECA). In our study, we followed Björkstén et al., and added a broad-spectrum cocktail of protease inhibitors. Despite all these precautions, it was shown that Mal d 1 is still not completely protected. Although we were unable to identify the exact cause, it was concluded that during the extraction of the I-cvs and/or their subsequent storage, Mal d 1 was more affected than was the case for the NL cvs. Such a difference was not observed for the stable apple LTP (Mal d 3), and to a far lesser extent (only 2-fold) for profilin (Mal d 4) suggesting that the processes affecting Mal d 1 are of limited relevance to the other allergens tested. Another observation supporting that it is very hard to preserve the native IgE-binding conformation of Mal d 1, was the SEC elution profile of purified PVPP^{plus}-Mal d 1 standard, which was dominated by aggregated Mal d 1. Whether the procedure for radiolabeling contributed to the degree of aggregation is not clear, because unlabeled Mal d 1 has not (yet) been analyzed by SEC. Interestingly, the mass variants of Mal d 1 detected by MS differed from each other by a mass similar to that of the proposed type of ligands for PR10 proteins, i.e. plant steroids [46]. This suggests that the absence of the natural ligand of Mal d 1 might be at the basis of aggregation and loss of immunoreactivity including IgE binding. Studies to identify the molecule responsible for the decrease in molecular mass are in progress. The ratio between Mal d 1 with and without hypothetical ligand in PVPP^{plus}-Mal d 1 observed in MS was not as extreme as between aggregated and non-aggregated observed in SEC. This suggests that radiolabeling might indeed increase aggregation.

A variable degree of preservation of allergenicity and immunoreactivity during extraction poses considerable problems to reliable assessment of allergenicity of apple cultivars. How do we interpret allergenicity data if we know that apple cultivars may differ in oxidase and protease content, may have variable levels of phenolic compounds, or may demonstrate different degrees of aggregation of Mal d 1? The fact that some assays have a preference for non-aggregated (native) Mal d 1 and others for aggregated Mal d 1 adds to the complexity. Although not very attractive for large screening exercises, *in vivo* prick-to-prick evaluation of the allergenicity of fruits might be more reliable because extraction can be avoided [18].

How useful are the Mal d 1 data that were published to date? In some reports the authors have used antibody-independent techniques like quantitative SDS-PAGE. Knowing the problems described in this study, such data might be more reliable. From these studies, levels of Mal d 1 were reported to be in the range of 10–50 µg/g [17, 47]. Data reported earlier [22] and in this study obtained with the Mal d 1-ELISA are at the low end of this range. Data obtained with the other three assays in the present study are significantly higher (up to around 500 µg/g). Which figures are closest to reality?

The problem we are facing here is complex. Essentially, we have two types of assays, those with preference for aggregated and those with preference for native Mal d 1. The latter assays were based on antibodies raised against Bet v 1 (Bet v 1-ELISA and cRIA) or on IgE antibodies induced upon natural exposure to Bet v 1. These antibodies are all cross-reactive to Mal d 1. Assays based on those antibodies showed good correlation (Spearman rank) amongst themselves but not to the Mal d 1-ELISA. A competitive ELISA based on another anti-Bet v 1 antibody BIP 1 [48] was performed for a selected number of cvs (*n* = 10). Results from this assay again showed good correlation with the three Bet v 1-based assays ($R_{\text{Spearman}} > 0.9$, 95% CI 0.63–0.97) but not the Mal d 1-ELISA ($R_{\text{Spearman}} = 0.5$, 95% CI 0.2–0.86).

Results obtained with the Bet v 1-ELISA were higher than those obtained with the cRIA. This most likely reflects the polyclonality of the ELISA covering a broader epitope spectrum (isoforms) than the mAb-based cRIA. The observation that the results obtained with RI were consistently the lowest implies that IgE epitopes are most sensitive during extraction and further processing.

The Mal d 1-ELISA was based on a mAb raised against predominantly aggregated Mal d 1 and a pAb against rMal d 1. It is not unlikely that this rMal d 1 was also largely aggregated, since crystallization attempts failed

[Z. Markovic-Housley, pers. commun.]. The Mal d 1-ELISA was not cross-reactive to Bet v 1, indicating that the aggregated state of Mal d 1 does not occur with natural Bet v 1. It is probably safe to assume that in an apple, Mal d 1 is in the native form. Aggregation is a result of extraction, extract handling, affinity purification, and/or radiolabeling. To assess allergenicity we would need to measure the sum of aggregated and native allergen. It is not known how much of the Mal d 1 in an apple extract is aggregated, or modified in any other way, and this might be different for each cultivar and/or extraction. This is further complicated by the fact that Mal d 1 standards have variable ratios of the different forms of Mal d 1 as well.

In summary, this study has demonstrated that immunoassay-based data on allergenicity of fruits containing labile PR10 allergens have to be interpreted with caution. On the basis of such data, it is difficult to produce a re-

liable ranking of cvs or even a convincing range of Mal d 1 levels within which most cvs fall. Immunoassays that do not distinguish between native and modified (aggregated) Mal d 1 are needed. Nevertheless, our study has given new leads for the relation between immunoreactivity and modifications of Mal d 1. This knowledge will facilitate improvement of methods for the detection of Mal d 1 and related allergens.

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References

- ▶ 1 Ebner C, Hirschehr R, Bauer L, Breiteneder H, Valenta R, Ebner H, Kraft D, Scheiner O: Identification of allergens in fruits and vegetables: IgE cross-reactivities with the important birch pollen allergens Bet v 1 and Bet v 2 (birch profilin). *J Allergy Clin Immunol* 1995;95:962–969.
- ▶ 2 Calkhoven PG, Aalbers M, Koshte VL, Pos O, Oei HD, Aalberse RC: Cross-reactivity among birch pollen, vegetables and fruits as detected by IgE antibodies is due to at least three distinct cross-reactive structures. *Allergy* 1987;42:382–390.
- ▶ 3 Breiteneder H, Hassfeld W, Pettenburger K, Jarolim E, Breitenbach M, Rumpold H, Kraft D, Scheiner O: Isolation and characterization of messenger RNA from male inflorescences and pollen of the white birch (*Betula verrucosa*). *Int Arch Allergy Appl Immunol* 1988;87:19–24.
- ▶ 4 Lahti A, Björkstén F, Hannuksela M: Allergy to birch pollen and apple, and cross-reactivity of the allergens studied with the RAST. *Allergy* 1980;35:297–300.
- ▶ 5 Valenta R, Duchene M, Ebner C, Valent P, Sillaber C, Deviller P, Ferreira F, Tejkl M, Edelmann H, Kraft D, et al: Profilins constitute a novel family of functional plant pan-allergens. *J Exp Med* 1992;175:377–385.
- ▶ 6 Karamloo F, Wangorsch A, Kasahara H, Davin LB, Haustein D, Lewis NG, Vieths S: Phenylcoumaran benzylic ether and isoflavonoid reductases are a new class of cross-reactive allergens in birch pollen, fruits and vegetables. *Eur J Biochem* 2001;268:5310–5320.
- ▶ 7 Fernandez-Rivas M, van Ree R, Cuevas M: Allergy to Rosaceae fruits without related pollinosis. *J Allergy Clin Immunol* 1997;100:728–733.
- ▶ 8 Pastorello EA, Pravettoni V, Farioli L, Spano M, Fortunato D, Monza M, Giuffrida MG, Rivolta F, Scibola E, Ansaloni R, Incorvaia C, Conti A, Ortolani C: Clinical role of a lipid transfer protein that acts as a new apple-specific allergen. *J Allergy Clin Immunol* 1999;104:1099–1106.
- ▶ 9 Sanchez-Monge R, Lombardero M, Garcia-Selles FJ, Barber D, Salcedo G: Lipid-transfer proteins are relevant allergens in fruit allergy. *J Allergy Clin Immunol* 1999;103:514–519.
- ▶ 10 Hsieh LS, Moos M Jr, Lin Y: Characterization of apple 18 and 31 kD allergens by microsequencing and evaluation of their content during storage and ripening. *J Allergy Clin Immunol* 1995;96:960–970.
- ▶ 11 Krebitz M, Wagner B, Ferreira F, Peterbauer C, Campillo N, Witty M, Kolarich D, Steinkellner H, Scheiner O, Breiteneder H: Plant-based heterologous expression of Mal d 2, a thaumatin-like protein and allergen of apple (*Malus domestica*), and its characterization as an antifungal protein. *J Mol Biol* 2003;329:721–730.
- ▶ 12 Ortolani C, Spano M, Pastorello E, Bigi A, Ansaloni R: The oral allergy syndrome. *Ann Allergy* 1988;61:47–52.
- ▶ 13 Van Ree R: The oral allergy syndrome; in Amin S, Lahti A, Maibach HI (eds): *Contact Urticaria Syndrome*. Boca Raton, CRC Press, 1997, pp 289–299.
- ▶ 14 Hoffmann-Sommergruber K: The SAFE Project: Plant Food Allergies: Field to Table Strategies for Reducing Their Incidence in Europe. *Allergy* 2005;60:436–442.
- ▶ 15 Pastorello EA, Vieths S, Pravettoni V, Farioli L, Trambaioli C, Fortunato D, Luttkopf D, Calamari M, Ansaloni R, Scibilia J, Ballmer-Weber BK, Poulsen LK, Wutrich B, Hansen KS, Robino AM, Ortolani C, Conti A: Identification of hazelnut major allergens in sensitive patients with positive double-blind, placebo-controlled food challenge results. *J Allergy Clin Immunol* 2002;109:563–570.
- ▶ 16 Vieths S, Jankiewicz A, Schoning B, Aulepp H: Apple allergy: the IgE-binding potency of apple strains is related to the occurrence of the 18-kDa allergen. *Allergy* 1994;49:262–271.
- ▶ 17 Son DY, Scheurer S, Hoffmann A, Haustein D, Vieths S: Pollen-related food allergy: cloning and immunological analysis of isoforms and mutants of Mal d 1, the major apple allergen, and Bet v 1, the major birch pollen allergen. *Eur J Nutr* 1999;38:201–215.
- ▶ 18 Bolhaar ST, Van de Weg WE, van RR, Gonzalez-Mancebo E, Zuidmeer L, Bruijnzeel-Koomen CA, Fernandez-Rivas M, Jansen J, Hoffmann-Sommergruber K, Knulst AC, Gilissen LJ: In vivo assessment with prick-to-prick testing and double-blind, placebo-controlled food challenge of allergenicity of apple cultivars. *J Allergy Clin Immunol* 2005;116:1080–1086.
- ▶ 19 Van Loon LCYAMG: Protease activity and pathogenesis-related proteins in virus-infected Samsun NN tobacco leaves. *Plant Sci* 2006;63:141.

- 20 Linthorst HJM: Pathogenesis-related proteins of plants. *Crit Rev Plant Sci* 2006;10: 123.
- ▶ 21 Ernst D, Schraudner M, Langebartels C, Sander mann H Jr: Ozone-induced changes of mRNA levels of β 1,3-glucanase, chitinase and 'pathogenesis-related' protein 1b in tobacco plants. *Plant Mol Biol* 1992;20:673–682.
- ▶ 22 Marzban G, Puehringer H, Dey R, Brynda S, Ma Y, Martinelli A, Zaccarini M, van der Weg E, Housley Z, Kolarich D: Localisation and distribution of the major allergens in apple fruits. *Plant Sci* 2005;169:387–394.
- ▶ 23 Björkstén F, Halmepuro L, Hannuksela M, Lahti A: Extraction and properties of apple allergens. *Allergy* 1980;35:671–677.
- ▶ 24 Akkerdaas JH, van Ree R, Aalbers M, Stapel SO, Aalberse RC: Multiplicity of cross-reactive epitopes on Bet v 1 as detected with monoclonal antibodies and human IgE. *Allergy* 1995;50:215–220.
- ▶ 25 Lind SE, Janmey PA, Chaponnier C, Herbert TJ, Stossel TP: Reversible binding of actin to gelsolin and profilin in human platelet extracts. *J Cell Biol* 1987;105:833–842.
- 26 Ma Y, Zuidmeer L, Bohle B, Bolhaar STH, Gadermaier G, Gonzalez-Mancebo E, Fernandez-Rivas M, Knulst AC, Himly M, Asero R, Ebner C, van Ree R, Ferreira F, Breiteneder H, Hoffmann-Sommergruber K: Characterization of recombinant Mal d 4 and its application for component-resolved diagnosis of apple allergy. 2006, in press.
- ▶ 27 Steinbuch M, Audran R: The isolation of IgG from mammalian sera with the aid of caprylic acid. *Arch Biochem Biophys* 1969;134: 279–284.
- ▶ 28 Van Ree R, van Leeuwen WA, van den BM, Weller HH, Aalberse RC: IgE and IgG cross-reactivity among Lol p I and Lol p II/III. Identification of the C-termini of Lol p I, II, and III as cross-reactive structures. *Allergy* 1994;49:254–261.
- ▶ 29 Zuidmeer L, van Leeuwen WA, Kleine BI, Cornelissen J, Bulder I, Rafalska I, Tellez BN, Akkerdaas JH, Asero R, Fernandez RM, Gonzalez ME, van Ree R: Lipid transfer proteins from fruit: cloning, expression and quantification. *Int Arch Allergy Immunol* 2005;137:273–281.
- ▶ 30 Van Ree R, Voitenko V, van Leeuwen WA, Aalberse RC: Profilin is a cross-reactive allergen in pollen and vegetable foods. *Int Arch Allergy Immunol* 1992;98:97–104.
- ▶ 31 Aalberse RC, Koshte V, Clemens JG: Immunoglobulin E antibodies that cross-react with vegetable foods, pollen, and *Hymenoptera* venom. *J Allergy Clin Immunol* 1981;68: 356–364.
- ▶ 32 Van Ree R, Clemens JG, Aalbers M, Stapel SO, Aalberse RC: Characterization with monoclonal and polyclonal antibodies of a new major allergen from grass pollen in the group I molecular weight range. *J Allergy Clin Immunol* 1989;83:144–151.
- ▶ 33 Moreno FJ, Jenkins JA, Mellon FA, Rigby NM, Robertson JA, Wellner N, Clare Mills EN: Mass spectrometry and structural characterization of 2S albumin isoforms from Brazil nuts (*Bertholletia excelsa*). *Biochim Biophys Acta* 2004;1698:175–186.
- ▶ 34 Ortolani C, Ispano M, Pastorello EA, Ansaloni R, Magri GC: Comparison of results of skin prick tests (with fresh foods and commercial food extracts) and RAST in 100 patients with oral allergy syndrome. *J Allergy Clin Immunol* 1989;83:683–690.
- ▶ 35 Rudeschko O, Fahlbusch B, Henzgen M, Schlenvoigt G, Herrmann D, Jager L: Optimization of apple allergen preparation for in vivo and in vitro diagnostics. *Allergy* 1995; 50:262–268.
- ▶ 36 Loomis WD: Overcoming problems of phenolics and quinones in the isolation of plant enzymes and organelles. *Methods Enzymol* 1974;31:528–544.
- ▶ 37 Rudeschko O, Fahlbusch B, Henzgen M, Schlenvoigt G, Herrmann D, Vieths S, Jager L: Investigation of the stability of apple allergen extracts. *Allergy* 1995;50:575–580.
- ▶ 38 Deluze C, Dunoyer-Geindre S, Girard JP: Comparative analysis of lyophilized fresh fruits and vegetables with commercial extracts for skin testing of patients with food allergy. *J Invest Allergol Clin Immunol* 1991;1:53–57.
- ▶ 39 Dreborg S, Foucard T: Allergy to apple, carrot and potato in children with birch pollen allergy. *Allergy* 1983;38:167–172.
- ▶ 40 Vieths S, Hoffmann A, Holzhauser T, Muller U, Reindl J, Hausteiner D: Factors influencing the quality of food extracts for in vitro and in vivo diagnosis. *Allergy* 1998;53:65–71.
- ▶ 41 Jensen-Jarolim E, Wiedermann U, Ganglberger E, Zurcher A, Stadler BM, Boltz-Nitulescu G, Scheiner O, Breiteneder H: Allergen mimotopes in food enhance type I allergic reactions in mice. *FASEB J* 1999;13: 1586–1592.
- ▶ 42 Asero R, Mistrello G, Roncarolo D, de Vries SC, Gautier MF, Ciurana CL, Verbeek E, Mohammadi T, Knul-Brettlova V, Akkerdaas JH, Bulder I, Aalberse RC, van Ree R: Lipid transfer protein: a pan-allergen in plant-derived foods that is highly resistant to pepsin digestion. *Int Arch Allergy Immunol* 2000; 122:20–32.
- ▶ 43 Scheurer S, Lauer I, Foetisch K, Moncin MS, Retzek M, Hartz C, Enrique E, Lidholm J, Cistero-Bahima A, Vieths S: Strong allergenicity of Pru av 3, the lipid transfer protein from cherry, is related to high stability against thermal processing and digestion. *J Allergy Clin Immunol* 2004;114:900–907.
- ▶ 44 Asero R, Mistrello G, Roncarolo D, Amato S, Falagiani P: Analysis of the heat stability of lipid transfer protein from apple. *J Allergy Clin Immunol* 2003;112:1009–1011.
- ▶ 45 Sancho AI, Rigby NM, Zuidmeer L, Asero R, Mistrello G, Amato S, Gonzalez-Mancebo E, Fernandez-Rivas M, van RR, Mills EN: The effect of thermal processing on the IgE reactivity of the non-specific lipid transfer protein from apple, Mal d 3. *Allergy* 2005;60: 1262–1268.
- ▶ 46 Markovic-Housley Z, Degano M, Lamba D, Roepenack-Lahaye E, Clemens S, Susani M, Ferreira F, Scheiner O, Breiteneder H: Crystal structure of a hypoallergenic isoform of the major birch pollen allergen Bet v 1 and its likely biological function as a plant steroid carrier. *J Mol Biol* 2003;325:123–133.
- ▶ 47 Vieths S, Schoening B, Jankiewicz A: Occurrence of IgE binding allergens during ripening of apple fruits. *Food Agric Immunol* 2006;5:93–105.
- ▶ 48 Laffer S, Vangelista L, Steinberger P, Kraft D, Pastore A, Valenta R: Molecular characterization of Bip 1, a monoclonal antibody that modulates IgE binding to birch pollen allergen, Bet v 1. *J Immunol* 1996;157:4953–4962.