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In vitro and in vivo Characterization of Hazelnut Skin Prick Test Extracts

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Abstract

Rationale: Hazelnut allergy ranks among the most frequently observed food allergies. Clinical symptoms range from the oral allergy syndrome to life threatening anaphylaxis. Diagnosis of hazelnut allergy partially relies on *in vivo* testing by means of skin prick testing (SPT). The aim of this study was to characterize hazelnut SPT extracts both *in vitro* and *in vivo*. **Methods:** Hazelnut SPT extracts were investigated for protein concentration and composition. The major hazelnut allergen Cor a 1, lipid transfer protein (LTP) and thaumatin-like-protein (TLP) were monitored by competitive RIA and immunoblotting. SPT extracts (n=6) were analyzed for skin reactivity and the correlation between the SPT extract protein concentration and the mean skin reactivity (HEIC) was determined in a group of hazelnut-allergic patients (n=30). For one SPT extract, the threshold level for Cor a 1 was determined in Cor a 1-monosensitized patients (n=5). **Results:** Protein concentrations ranged from 0.2-14 mg/ml. Although some proteins were present in most extracts (bands at 10, 22-28, 32 and around 48 kDa), clear differences in composition were observed (both intra- and inter-variability). The concentration of the major hazelnut allergen Cor a 1 differed up to a factor 50 (0.6-32 µg/ml). LTP was virtually absent in 3/9 SPT extracts and variable quantities of TLP were detected by immunoblotting. Some patients (6/30) had a false-negative SPT with 3/6 SPT extracts. There was a clear correlation between the protein concentration and the mean HEIC ($R_{\text{Pearson}}=0.87$). The threshold level for Cor a 1 was ± 3.2 ng/ml as assessed with one of the products investigated. **Conclusions:** Heterogeneous protein concentration/composition of SPT extracts results in variable skin test responses. The absence of potentially severe allergens like LTP may lead to false-negative SPT results that jeopardize a patient's safety. From these results it can be concluded that there is a strong need for standardization of products for SPT.

Introduction

Tree nut allergy has an estimated prevalence of 0.7% (1-3) (in North America and Western Europe). In general, hazelnut is known to cause rather mild adverse reactions (OAS), but also more severe reactions upon consumption are observed, including anaphylactic shock.

Hazelnut allergy often occurs as a result of primary sensitization to birch pollen. This is due to IgE directed against the major birch pollen allergen Bet v 1 that crossreacts with its homologue found in hazelnut, Cor a 1. These allergens, belonging to a family of pathogenesis-related (PR10) proteins (4) are known to cause only mild symptoms. Due to their labile properties they cannot easily reach the gastro-intestinal tract (GIT) in an IgE binding conformation, and are consequently not likely to evoke more severe systemic symptoms. However, other allergens, like the lipid transfer protein (LTP) (5,6), 2S albumin (7,8) and thaumatin-like-protein (TLP) (9), have the physiological properties to survive the acidic and proteolytic environment of the GIT. IgE against these allergens is frequently associated with more severe symptoms (10). In order to correctly identify patients at risk of having severe adverse reactions after hazelnut consumption, it is important to use skin prick test extracts containing sufficient quantities of all relevant stable allergens.

Although the double-blind placebo-controlled food challenge (DBPCFC) is considered as the gold standard (11) of food allergy diagnosis, the skin prick test is the most widely applied diagnostic tool. It can be assumed that differences in manufacturing SPT extracts may lead to different diagnostic performance.

This study was focused on the immunochemical characterization of commercially available hazelnut SPT extracts. In order to compare the diagnostic performance of these extracts, a panel of hazelnut-allergic patients was tested by skin prick. Cor a 1 threshold concentrations were determined for Cor a 1 monosensitized patients.

Material & Methods

Patients

Dutch hazelnut allergic patients (n=30) with a convincing history of acute hazelnut-related symptoms (14/30 patients were tested by DBPCFC and 100% reacted positive) and IgE against hazelnut (≥ 0.7 kU/ml) were recruited for SPT. Hazelnut-related symptoms ranged from OAS to systemic reactions like urticaria and nausea and faintness. The study was approved by the local ethical committee.

Extracts

Commercially available hazelnut SPT extracts (n=9) were purchased from nine different manufacturers (A-I). Of each manufacturer, two different batches were obtained. The protein concentration of each extract was determined (Pierce, Rockford, IL, USA) after extensive dialysis. Results were adjusted for changes in volume. An in-house hazelnut extract (IHE) was prepared according to the method described by Björkstén *et al.* (12). The protein composition was studied by SDS-PAGE/silver staining

using an Excelgel SD Western blotting wa 4-12% gel (Novex. S

Human serum samp

Sera (n=5) from hazelnut consumption (ting) were used in h

Mab 5H8 directed detect Cor a 1. Rab anti-apple TLP (9) v

Competitive RIA

Cor a 1 was meas 50 μ l (6.7 ng) mAb SPT extract in a fina mM EDTA, 0.3% b rose-coupled goat a Netherlands), ^{125} I-ra final volume of 850 was measured in a curve of mAb 5H8.

Immunoblotting

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Immunoblots of specific rabbit anti sheep anti-rabbit I KODAK X-AR fil

In vivo reactivity of

SPTs were perfor zed prick needle (A (A,B,D,G,H and I)

Histamine dihyd erol diluent of the were expressed rel equivalent intracut divided by that of < 3 mm for the neg regarded negative. week.

using an Excelgel SDS gradient 8-18% (Amersham Pharmacia Biotech, Uppsala, Sweden). Western blotting was performed after SDS-PAGE on a Novex Xcell II system using a 4-12% gel (Novex, San Diego, CA, USA), according to the manufacturer's description.

Human serum samples and allergen-specific monoclonal/polyclonal antibodies

Sera ($n=5$) from Dutch patients with positive SPT, clear clinical symptoms after hazelnut consumption and IgE exclusively against Cor a 1 (as judged by immunoblotting) were used in hazelnut RAST-inhibition with Bet v 1.

Mab 5H8 directed against Bet v 1 (13) and crossreactive to Cor a 1 was used to detect Cor a 1. Rabbit anti-carrot LTP (14) was used to detect hazelnut LTP. Rabbit anti-apple TLP (9) was used to detect hazelnut TLP.

Competitive RIA

Cor a 1 was measured by competitive RIA in batch 2 of all manufacturers. In short, 50 μ l (6.7 ng) mAb 5H8 against Bet v 1 was incubated with serial dilutions of hazelnut SPT extract in a final volume of 100 μ l PBS/AT (PBS/AT: Phosphate-buffered saline, 10 mM EDTA, 0.3% bovine serum albumin, 0.1% TWEEN 20). After two hours, Sepharose-coupled goat antibodies against mouse IgG (GM 17D, Sanquin, Amsterdam, The Netherlands), ^{125} I-radiolabeled Bet v 1 and 500 μ l PBS/AT were added resulting in a final volume of 850 μ l. After overnight incubation and washing, bound radioactivity was measured in a γ -counter. Cor a 1 concentrations were calculated using a standard curve of mAb 5H8 affinity purified Cor a 1.

Immunoblotting

Hazelnut immunoblot (using the hazelnut IHE) was cut in strips 3 mm wide. Strips were subsequently incubated with 150 μ l human serum in a final volume of 3 ml PBS/AT and incubated overnight. After washing, bound IgE was detected using ^{125} I-radiolabeled sheep anti-human IgE.

Immunoblots of hazelnut SPT extracts (A-I) were monitored for TLP and LTP using specific rabbit antisera. Bound rabbit antibodies were detected using ^{125} I-radiolabeled sheep anti-rabbit IgG. Radiolabel bound was detected by autoradiography, using KODAK X-AR films.

***In vivo* reactivity of hazelnut SPT extracts**

SPTs were performed on the flexor aspect of the patients' forearm with a standardized prick needle (ALK Lancet) according to Dreborg, using the first batch of 6 extracts (A,B,D,G,H and I).

Histamine dihydrochloride (10 mg/ml) was used as a positive control and the glycerol diluent of the SPT-extracts as a negative control (ALK-Abellö). SPT wheal sizes were expressed relative to the wheal induced by histamine, the so-called histamine equivalent intracutaneous test (HEIC). The mean diameter of the allergen wheal is divided by that of the histamine wheal. All patients fulfilled the criteria of a wheal < 3 mm for the negative control and ≥ 5 mm for histamine. A HEIC of 0.25 or less was regarded negative. Prior to the skin tests, patients discontinued antihistamines for one week.

RAST-inhibition

For hazelnut RAST-inhibition, IHE was coupled to Sepharose (4 mg protein per 100 mg CNBr-activated Sepharose, Amersham Pharmacia Biotech). Serum (50 µl) was pre-incubated with 100 µg/ml Bet v 1 (50 µl). After 2 hours, 1.5 mg hazelnut Sepharose was added. Subsequently, RAST was performed as described elsewhere (15).

Results

Protein composition of hazelnut SPT extracts.

Protein concentrations ranged from 0.2-14 mg/ml (inter-company variation). Intra-company variation was limited (factor <2, Table 1). Protein composition was analyzed by SDS-PAGE-silverstaining (not shown). Although some proteins were present in most extracts (bands at 10, 22-28, 32 and around 48 kDa), clear differences in composition were observed. Intra-variability was observed for extract B (extra band at 58 kDa in batch 2) and extract D (extra band at 10 kDa in batch 2).

Table 1. Analysis of nine hazelnut skin prick test extracts.

SPT manufacturer	Protein B1* (µg/ml)	Protein B2* (µg/ml)	Ratio Protein B1/B2	Cor a 1 B2 (µg/ml)	Cor a 1 B2/total Protein (w/w%)
A	751	1003	0.72	3.2	0.32
B	250	217	1.15	0.6	0.28
C	2700	1904	1.42	3.3	0.17
D	3340	3360	0.99	1.5	0.05
E	230	389	0.5	6.1	1.57
F	4800	4995	0.96	10.9	0.23
G	3250	4810	0.68	11	0.22
H	14091	13440	1.05	10.9	0.23
I	715	970	0.73	32	0.24

B1*: batch 1, B2*: batch 2

Allergen measurement

Competitive RIA demonstrated Cor a 1 concentrations ranging from 0.6 to 32 µg/ml (Table 1) in hazelnut SPT extracts. The ratio Cor a 1/total protein varied from 0.05-1.57% w/w, mean 0.38% ($\pm 0.17\%$ SE).

Monitoring 9 hazelnut SPT extracts with rabbit anti-carrot LTP showed the virtual absence of hazelnut LTP in 3/9 extracts (Figure 1). Immunoblot with rabbit anti-apple TLP revealed that extract C contained hardly any detectable TLP. In the other extracts,

either monomeric or dimeric anti-TLP (data not shown).

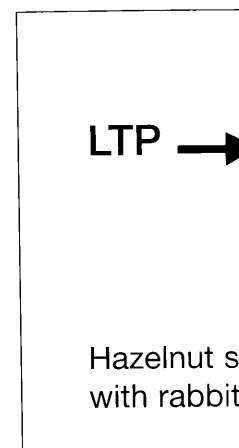
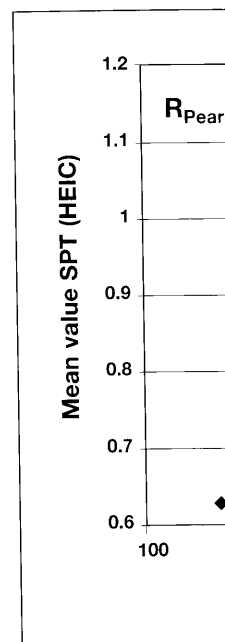


Fig. 1. Hazelnut SPT extracts tested with rabbit anti-carrot LTP.

In vivo reactivity of hazelnut SPT extracts

Hazelnut-allergic patients (A, B, D, G, H and I) were tested with different protein concentrations of hazelnut SPT extracts (Table 2).



4 mg protein per
Serum (50 µl) was
hazelnut Sepharose
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variation). Intra-
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extra band at 58 kDa

either monomeric or dimeric TLP or a combination of both was recognized by rabbit anti-TLP (data not shown).

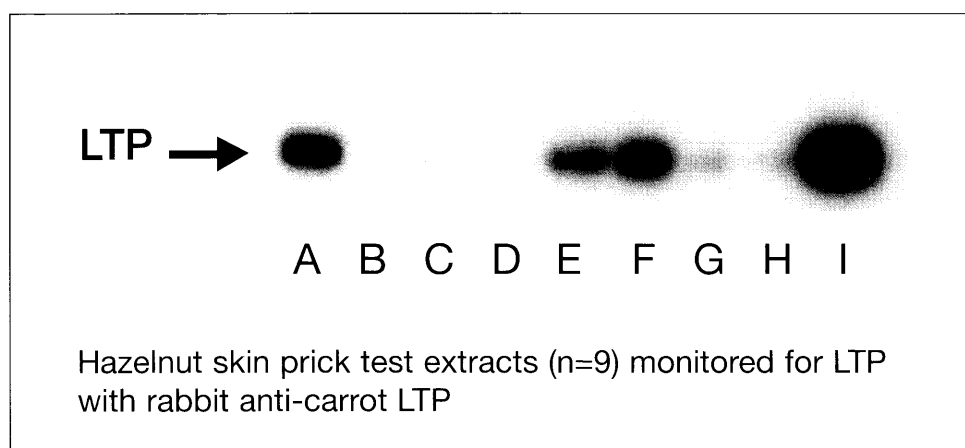


Fig. 1. Hazelnut SPT extract immunoblots monitored for LTP.

B2
Cor a 1 B2/total
Protein (w/w%)

0.32

0.28

0.17

0.05

1.57

0.23

0.22

0.23

0.24

from 0.6 to 32 µg/ml
varied from 0.05-

showed the virtual
with rabbit anti-apple
the other extracts,

In vivo reactivity of hazelnut SPT extracts

Hazelnut-allergic patients (n=30) were tested by SPT with hazelnut extracts A,B,D,G,H and I. Mean HEICs varied from 0.63-1.15. The observed correlation between protein concentration and skin reactivity (Figure 2) was $R_{\text{Pearson}}: 0.87$. False-negative SPT results were observed for 6/30 patients with 3/6 SPT extracts (B, G and I, Table 2).

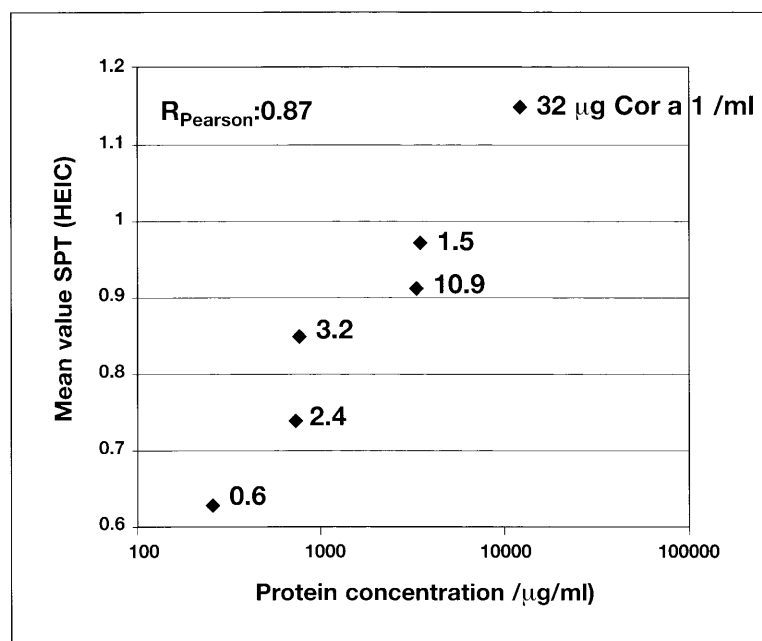


Fig. 2.
Correlation of
protein concentra-
tion in extracts
vs SPT results in
30 patients.

Table 2. Mean HEICs determined for 6 patients with 6 hazelnut SPT extracts. For each SPT extract, the Cor a 1 concentration is indicated.

Patient	DBPCFC (Y/N)	Pos DBPCFC	Cor a 1 (mg/ml)						IgE specificity*
			3.2	0.6	3.3	10.9	32	2.4	
			Extr. A	Extr. B	Extr. D	Extr. G	Extr. H	Extr. I	
1	N		0.9 2	0.0 0	1.3 6	0.8 2	1.4 6	0.0 0	Cor a 1
2	Y	Y	0.9 4	0.5 0	0.5 6	0.6 3	0.9 4	0.0 0	Not defined
3	Y	Y	0.6 2	0.0 0	0.7 7	0.7 7	1.1 5	0.6 2	Cor a 1
4	N		0.4 3	0.4 3	0.6 4	0.0 0	0.5 0	0.0 0	Not defined
5	Y	Y	0.6 7	0.0 0	0.4 0	0.4 7	0.6 0	0.4 0	Cor a 1
6	Y	Y	1.0 0	0.0 0	0.9 1	1.4 5	1.3 6	0.9 1	Cor a 1

* as judged by immunoblotting

Determination Cor a 1 concentration threshold levels in SPT extract A

To determine a threshold for Cor a 1 in SPT, extract of manufacturer A, batch 2, was tested on five patients selected for monospecificity to Cor a 1. Mono-specificity was assessed by immunoblotting and hazelnut RAST-inhibition with Bet v 1 (Figure 3). The extract used contained 3.2 µg/ml Cor a 1 as determined by competitive RIA (total protein 1003 µg/ml).

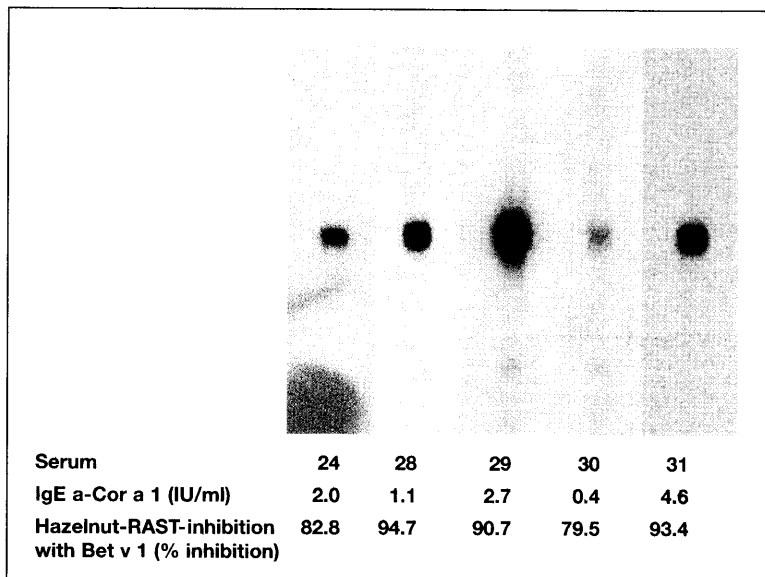


Fig. 3. Hazelnut immunoblot and RAST (inhibition) results.

Using a cut-off value of 3.2 ng/ml Cor a 1 (Fig. 4) tested in only one HEIC.

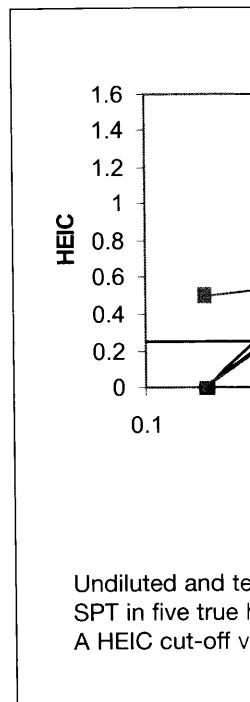


Fig. 4. Determination of Cor a 1 concentration threshold levels in SPT extract A.

Discussion

In vivo diagnosis of hazelnut allergy is of great importance to ensure a high quality from the hazelnut protein/allergen component. Furthermore, skin reactions to hazelnut allergen, were determined.

SDS-PAGE/silver staining showed clear differences between the observed differences in the results of picking the nuts.

The most relevant allergen is Cor a 1. In most cases, a high level of IgE antibodies directed against Cor a 1 react with other Bet v 1 allergens.

For each SPT extract, the

Using a cut-off value of 0.25 HEIC, 5/5 patients still demonstrated a positive SPT at 3.2 ng/ml Cor a 1 (Figure 4). Lowering the SPT extract A concentration tenfold resulted in only one HEIC > 0.25.

2.4

Extr.	IgE
I	specificity*
0.0	Cor a 1
0	
0.0	Not defined
0	
0.6	Cor a 1
2	
0.0	Not defined
0	
0.4	Cor a 1
0	
0.9	Cor a 1
1	

act A

ater A, batch 2, was
mono-specificity was
v 1 (Figure 3). The
ive RIA (total pro-

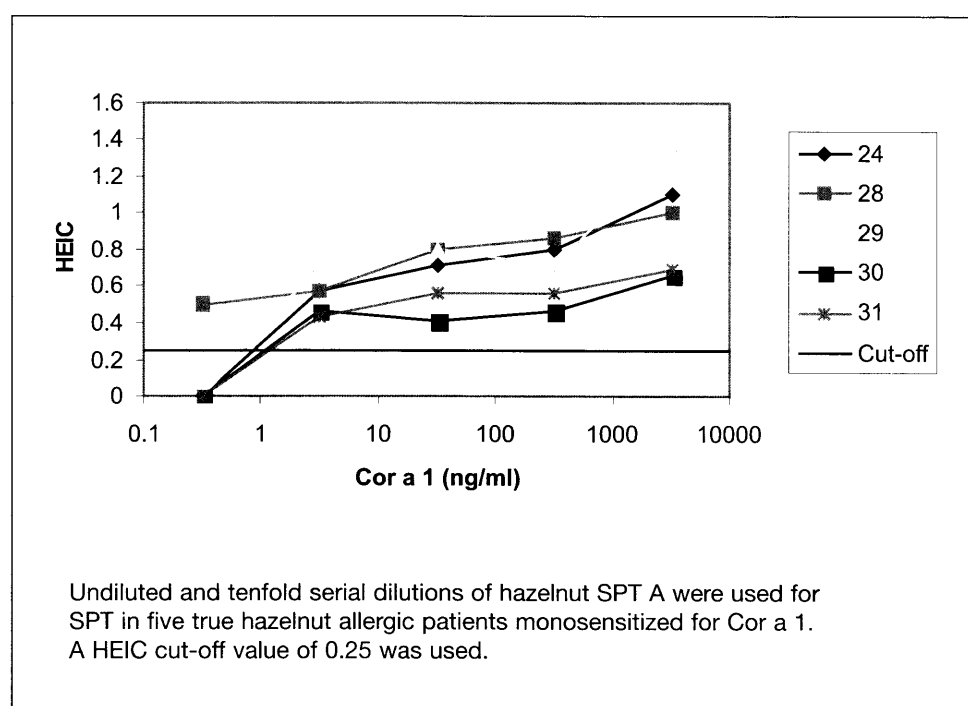


Fig. 4. Determination of the Cor a 1 threshold concentration in SPT extract A.

Discussion

In vivo diagnosis of food allergy is performed by skin prick testing. Therefore, it is of great importance to use extracts that contain all relevant allergens and that have a constant quality from batch to batch. This study focused on the measurement of protein/allergen composition and concentration in hazelnut skin prick test extracts. Furthermore, skin reactivity and threshold levels of Cor a 1, the major hazelnut allergen, were determined.

SDS-PAGE/silver staining performed with the nine hazelnut extracts (batch 1 and 2) showed clear differences in composition. Also, intra-variability was observed. The observed differences can be explained by a broad spectrum of different factors. Time of picking the nuts, different varieties, post-harvest treatment, extraction method etc.

The most relevant hazelnut allergen for North America and Western Europe is Cor a 1. In most cases, a primary birch pollen sensitization is characterized by the induction of IgE antibodies directed against Bet v 1. IgE against Bet v 1 can subsequently cross-react with other Bet v 1-homologous proteins like Cor a 1.

Fig. 3.
Hazelnut immuno-
blot and RAST
(inhibition) results.

A competitive RIA showed a 50-fold difference in Cor a 1 concentration in the nine hazelnut SPT extracts. However, when the ratio of Cor a 1/total protein was calculated, 7/9 extracts showed surprisingly comparable ratios (0.17-0.32). Because Cor a 1 is thought to be a labile protein like most Bet v 1-related fruit allergens, a higher degree of variability was expected.

Hazelnut LTP is a stable allergen that can evoke more severe symptoms and was virtually absent in 3/9 SPT extracts. Insufficient quantities of this allergen in SPT extract may lead to false-negative SPT diagnosis. In North America and Western Europe, where most hazelnut-allergic patients are primarily sensitized by the major birch pollen allergen Bet v 1 and its homologues, IgE to hazelnut LTP is not frequently observed. However, hazelnut allergic individuals from the Mediterranean countries, like Spain, frequently demonstrate specific IgE antibodies against LTP only (personal communication Dr. F. Schocker, Forschungszentrum Borstel, Germany). The importance of LTP for fruit- and nut-allergic patients from this hemisphere has been reported by several groups. This stresses the need for standardization of this clinically relevant allergen.

Although TLP has not been described in literature as extensively as LTP, this stable protein may play a role in evoking severe allergic reactions. The variable quantity and appearance (monomer/dimer) of this protein in hazelnut extracts also indicates the need for further standardization.

The *in vivo* performance of the SPT extracts was evaluated. Application of 6 different hazelnut SPT extracts in a group (n=30) hazelnut allergic patients showed a clear correlation ($R_{\text{Pearson}}: 0.87$) between the protein concentration of the SPT extract and the observed mean HEIC. This might implicate that insufficient quantities of hazelnut protein applied to the skin result in false-negative SPT results. In a control panel of 4 non-atopic and 3 atopic patients, none of the SPT extracts gave a false-positive SPT, including extract H (14 mg/ml protein). The SPT extracts that gave false-negative results were extracts B, G and I. Because the patient group (n=30) was selected from a Dutch population, most patients were primarily sensitized by Bet v 1/Cor a 1. Four out of six of the patients with false-negative SPTs indeed were Cor a 1-positive. For the two remaining patients this was not tested due to lack of serum. Sufficient quantities of Cor a 1 in the SPT extracts therefore is a prerequisite to perform reliable SPT in such patients. Under-representation of this allergen may be the cause of false-negative SPT diagnoses. Although extracts B and I demonstrated the lowest Cor a 1 concentrations of all extracts applied (0.6 mg/ml and 2.4 µg/ml respectively), all three products contained much more Cor a 1 than the threshold of 3.2 ng/ml. It is unclear why these extracts with at least 100 times the threshold concentration for Cor a 1 gave false-negative SPT results. Most likely, the IgE reactivity of Cor a 1 in these products is strongly decreased with the epitope recognized by the monoclonal antibody used in the competitive RIA being unaffected.

In summary, this study strongly indicates the need for further standardization of SPT extracts. The (virtual) absence or under-representation of relevant allergens like Cor a 1 or hazelnut LTP in hazelnut SPT extracts may lead to false-negative *in vivo* diagnoses. Especially in countries where LTP is a dominant allergen, such false-negative result can jeopardize a patient's safety.

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centration in the nine
protein was calculated.
Because Cor a 1 is
sens, a higher degree

symptoms and was
allergen in SPT extract
and Western Europe,
the major birch pollen
frequently observed.
countries, like Spain,
personal communi-
importance of LTP
reported by several
relevant allergen.

as LTP, this stable
variable quantity and
also indicates the

lication of 6 differ-
ents showed a clear
SPT extract and the
ies of hazelnut pro-
panel of 4 non-
sitive SPT, includ-
negative results were
from a Dutch popu-
out of six of the
the two remaining
es of Cor a 1 in the
T in such patients.
negative SPT diagno-
concentrations of all
products contained
these extracts with
false-negative SPT
strongly decreased
a competitive RIA

standardization of SPT
allergens like Cor a
tive *in vivo* diagno-
false-negative result

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