

Biochemical and immunological analysis of mould skin prick test solution: current status of standardization

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Summary

Background Sensitization prevalence to moulds reached from less than 10% in the general population to more than 25% in atopic and/or asthmatic subjects. To diagnose IgE-mediated mould sensitization, skin prick test (SPT) and specific IgE (sIgE) measurement are recommended. However, concordance of SPT and sIgE results is often less than 50% and standardization of the extracts is required to achieve reliable test results.

Objective The aim of our study was to analyse mould SPT solutions (SPTs) with respect to quantity and quality of protein, antigen and human IgE-binding content as a prerequisite for further *in vivo* studies.

Methods Commercial SPTs of *Alternaria alternata*, *Aspergillus fumigatus*, *Cladosporium herbarum* and *Penicillium chrysogenum* from six manufacturers as well as two in-house extracts from *Aspergillus versicolor* were investigated. Protein-, antigen- and IgE-binding contents were quantified by Bradford assay, sandwich ELISA and IgE-ImmunoCAP-inhibition tests. Protein composition and IgE and IgG binding were analysed by SDS-PAGE and immunoblotting, respectively.

Results Median protein concentrations were similar in all mould SPT extracts (90–110 µg/mL). In contrast, antigen contents and IgE-binding capacity showed a high variability with median antigen values from 4 to 118 µg/mL and IgE inhibition results between 30 to 95%. Whereas almost all SPTs of *A. alternata* and *A. versicolor* showed complete sIgE inhibition with mean values > 80%, only three extracts for *A. fumigatus*, two extracts for *C. herbarum* and none of the tested extracts for *P. chrysogenum* exceeded 50% sIgE reduction. Quantitative amounts of protein, antigenic and IgE-binding structures were not comparable with the quality of the corresponding protein or immunoblot pattern, with the exception of *A. alternata* SPTs.

Conclusions and Clinical Relevance Commercially available mould SPT extracts showed high variability raising the question of comparability and reliability of SPT results. Possible consequences for diagnostic test outcome will be investigated in the next step.

Keywords allergy, allergen content, mould allergen extracts, skin prick test, standardization
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Introduction

Moulds are a well-known source of environmental allergens. In the GA²len skin test study II [1], the sensitization prevalence of predominantly outdoor moulds, such as *Alternaria alternata* (*A. alternata*) and *Cladosporium herbarum* (*C. herbarum*), was about 9% and 5%, respectively. Clinically relevant allergic symptoms were found in 69% of the *A. alternata*-sensitized patients and in 62% of the *C. herbarum*-sensitized subjects. An example

of a predominantly indoor relevant mould, *Aspergillus* was reported with a sensitization prevalence of 4%. In other population-based studies [2–4], reported sensitization prevalence against mould allergens was also below 10%. However, in the case of asthma and especially severe asthma, mould sensitization prevalence [5–7] to a variety of moulds was much higher, and studies among atopic subjects demonstrated mould sensitization prevalence of 20 to 80% [8, 9]. Asthmatic patients show not only considerably higher sensitization rates, but

suffering dramatically from interaction with moulds (especially *Aspergillus fumigatus*, *A. fumigatus*) by means of reduced lung function, conducted by damaging effects on airway colonization with *A. fumigatus* [10] as well as airflow limitation and bronchiectasis in cases of severe asthma [11]. Another study demonstrated large improvements in quality of life in about 60% of patient suffering from severe asthma with fungal sensitization (SAFS) after oral antifungal therapy [12] indicating a direct link between fungal exposure and asthma.

Most studies investigating sensitization prevalence to indoor and outdoor allergens used *C. herbarum* and/or *A. alternata* as examples of 'outdoor' mould, and/or *A. fumigatus* as a sample of a primarily 'indoor' mould, mainly because these allergens are most commonly commercially available. However, these are limited examples of mould allergens because they are often not the only responsible and relevant allergen source, especially indoors in moisture-damaged dwellings. This raises the question whether the mould allergen test panel is sufficient if sensitization against currently unavailable mould species remains undetected. In several studies, the relationship between dampness in houses, mould and health effects is described [13–16]. *Aspergillus versicolor* (*A. versicolor*) is one of the typical indoor mould species used as an indicator of dampness in houses [17–20], but is not frequently tested in exposed subjects for sIgE sensitization, because commercial *A. versicolor* skin prick test extracts are rare or no longer available. In the German Environmental Survey for Children (GerESIV) of the Robert Koch Institute (www.uba.de/uba-info-medien/4176.html), more than 1500 (1538 – 1575) children were examined regarding mould sensitization and mould exposure. The sIgE test panel included four mould species that are especially relevant for indoor air and/or dampness in houses. These were *A. versicolor*, *Penicillium chrysogenum* (*P. chrysogenum*), *Wallemia sebi* (*W. sebi*) and *Eurotium spp.* Altogether, seven mould species were tested with sensitization rates of 4.8% for *A. alternata*, 5.0% for *P. chrysogenum*, 2.6% for *A. fumigatus*, 2.3% for *A. versicolor*, 2.1% for *C. herbarum*, 1.6% for *Eurotium spec.* and 0.2% for *W. sebi*. Airborne measurement (GerESIV) of mould exposure confirmed that *A. alternata* exposure occurs predominantly outdoors, whereas the mould species: *P. chrysogenum*, *A. fumigatus*, *A. versicolor*, *C. herbarum*, *Eurotium spec.* and *W. sebi* are more frequently found indoors.

In general, it is equally recommended to diagnose IgE-mediated sensitization by skin prick test and specific IgE measurement (www.dgaki.de/leitlinien; Allergo Journal 2009), but in the case of mould sensitization, the concordance between skin prick test and sIgE results was only 14–56% [21]. The heterogeneity of allergen test extracts in general is known and was also

described for mould test extracts [2, 21–25]. According to the CREATE study [22–24], standardization of all allergen extracts is essential to allow for comparability among study results and to warrant exact allergen doses in diagnostic and therapeutic test solutions. For allergen sources with dominant major allergens like birch (Bet v 1) and timothy grass (Phl p 5a), certified reference materials are available [26]. But if allergen sources do not have one or more dominant major allergens or are not investigated as intensively, evaluation of allergen content is difficult. In the case *A. alternata* and *A. fumigatus*, major allergens (Alt a 1 and Asp f 1), are well characterized. For other mould species several allergens are described but not with comparable clinical significance like major allergen Alt a 1. If no allergens were characterized for a mould species suggestions have been made to evaluate their allergenic potency using the sIgE-inhibition test, as well as by additional biochemical parameters [22, 27].

The aim of the present study was to analyse the biochemical properties of commercial skin prick test solutions, with emphasis on protein, antigen and allergen content for the frequently used mould species: *A. alternata*, *A. fumigatus*, *C. herbarum* and *P. chrysogenum*. In addition, an allergen extract for *A. versicolor* as indicator for indoor dampness, which is at present commercially unavailable, was prepared and investigated as well.

Material and methods

Skin prick test solution (SPTs) and mould extract preparations

Questionnaires were used to record the manufacturers of the mould skin prick test extracts for *A. alternata*, *A. fumigatus*, *C. herbarum/cladosporoides* and *P. chrysogenum* used by eighteen German allergological centres or practices in the autumn of 2011. In all medical centres, *A. alternata*, *A. fumigatus* and *C. herbarum* were tested ($n = 17$ SPT, $n = 1$ intracutaneous test (IC)), and in six practices, *P. chrysogenum* was tested as well ($n = 5$ SPT, $n = 1$ IC). The mould SPTs were most frequently purchased from Allergopharma (Reinbek, Germany) and ALK-Abelló (Wedel, Germany), but SPTs were also ordered from Bencard (Munich, Germany), HAL (Duesseldorf, Germany) and Lofarma (Willich, Germany). There was no record of purchase from Allergo (by Themocare, Moenchengladbach, Germany) or Stallergen (Kamp-Lintfort, Germany), but these companies were nevertheless included in the study. Table 1 summarizes mould SPT solutions purchased in 2011 that were used for biochemical analysis. Skin prick test solution for *A. alternata* were obtained from all seven manufacturers (No. A–G); all other mould SPTs could only be received from six of seven companies (see

Table 1. Analysed SPTs according to manufacturers and batch numbers

No.	Manufacturer	<i>A. alternata</i> (Aa) batch:	<i>A. fumigatus</i> (Af) batch:	<i>C. herbarum</i> (Ch) batch:	<i>P. chrysogenum</i> (Pc) batch:	<i>A. versicolor</i> (Av) batch:
A	ALK	D1667_DE03	E0242_DE03	E0470_DE02	D2491_DE02	—
B	Allergopharma	T9001604-DE	T9001783 -DE	T0002324-DE	T9001487-DE	—
C	Bencard	A12217NT	—	A12496LQ	A12057 LU	—
D	Lofarma	10041	A2611	C0411	09051	—
E	HAL	B1913046	B2009032	—	B1915011	—
F	Stallergen	93572	93222 (Mix)	X95282(Mix)	93832	—
G	Allerbio	0803297	901125 (Mix)	0903499 (Mix)	—	—
H	Allergon (Lyo)	—	—	—	—	AvA-11-09-26
I	Greer (Lyo)	—	—	—	—	AvG-11-09-27

Table 1). Additionally, skin prick test solutions for the indoor/dampness related mould, *A. versicolor*, were prepared in our laboratory (as in-house extract). Therefore, freeze-dried, non-viable material from *A. versicolor*, either from Allergon (Aengelholm, Sweden) or from Greer (Lenoir, NC, USA), was suspended in 0.45% NaCl. Suspensions were extracted by homogenization of the mould material in SK38 tubes containing glass/ceramic beads in Precellys24 (PeqLab, Erlangen, Germany) for three cycles of 20 s with 30 s break between each cycle at 6000 agitation per minute at 4°C, according to [28]. The extracts were then incubated for 10 min at 4°C and 5 min in an ultrasonic bath, centrifuged at 20 000 × g for 30 min at 4°C. Supernatants were transferred into new tubes and centrifuged again at 10 000 × g for 5 min at 4°C. Protein content was measured using the Bradford assay (Bio-Rad, Munich, Germany) with bovine serum albumin as the standard. The extract was concentrated 2-fold by lyophilizing and resuspending in 50% glycerol to a final solution of 0.9% NaCl / 50% glycerol. The mould extract was sterile-filtered with 0.45 µm Satorlab P20 (Satorius, Goettingen, Germany).

Electrophoresis and immunoblotting

Mould skin prick test extracts were separated according to the molecular weight of the proteins by SDS-PAGE using precasted 12% NuPAGE gels (Invitrogen, San Diego, CA, USA) and the Xcell II mini-cell unit of Novex, according to the manufacturer's instructions. For each skin prick test solution, the same volume (10 µL) was mixed with SDS sample buffer (Laemmli), boiled and applied for SDS-PAGE. For silver staining [29] as well as for immunoblotting, the same amount of SPT solution was used. For immunostaining, resolved proteins were first electrotransferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Eschborn, Germany) by semi-dry blotting using NuPAGE transfer buffer (Invitrogen) at 1 mA/cm² for 60 min at room temperature. Polyvinylidene difluoride blots were blocked in Tris-buffered saline (TBS) containing 1% bovine serum albumin (BSA) and 1% polyvinylpyrrolid-

one 40 (PVP40) for at least 6 h at room temperature. The membranes were either incubated for two hours with mould-specific polyclonal antibodies (pAb) diluted 1 : 1000 in TBS / 2% BSA / 0.1% Tween 20 to identify IgG-binding antigens or with specific human serum pool diluted to 2 kU_A/L. Nonspecific binding was eliminated by 4 × 5 min washing with 0.1% Tween 20 in TBS (TTBS). Specific IgG was detected by anti-rabbit IgG antibodies conjugated with alkaline phosphatase (Sigma), diluted 1 : 30 000 in 2% BSA/TTBS. Specific human IgE was detected with anti-human IgE antibodies conjugated with alkaline phosphatase (Sigma), diluted 1 : 1000 in buffer. Enzyme reactions were visualized by NBT/BCIP substrate tablets (Sigma).

Specific IgG rabbit antibodies and patient sera against mould

Aspergillus fumigatus-, *A. versicolor*-, *C. herbarum*- and *P. chrysogenum*-specific polyclonal antibodies were produced and purified as described previously [28, 30]. Human serum samples from three to six subjects with individual mould sensitization and suspected allergic symptoms were used for sIgE detection. Equal volumes of sera were mixed to generate IgE pool sera with the following sIgE concentrations: *A. alternata* pool (*n* = 5) 12.5 kU_A/L, *A. fumigatus* pool (*n* = 6) 12.3 kU_A/L, *C. herbarum* pool (*n* = 3) 14.4 kU_A/L, *P. chrysogenum* pool (*n* = 4) 4.0 kU_A/L and *A. versicolor* pool (*n* = 4) 2.5 kU_A/L. In addition, the human serum pool for *A. alternata* was tested on recombinant (r) Alt a 1 (m229) with 7.18 kU_A/L; and the *A. fumigatus* pool was tested according to rAsp f 1 (m218): 5.2 kU_A/L, rAsp f 2 (m219) 2.3 kU_A/L, rAsp f 3 (m220): 2.3 kU_A/L, rAsp f 4 (m221): 0.07 kU_A/L, and rAsp f 6 (m221): 0.39 kU_A/L. All sIgE tests were carried out using ImmunoCAP 250 system (Phadia, Freiburg, Germany).

Mould antigen and allergen quantification

Mould antigen concentrations were determined for *A. versicolor*, *A. fumigatus*, *C. herbarum* and

P. chrysogenum by polyclonal sandwich ELISA as described in [28, 30]. Each skin prick test solution was serially diluted to fit the measuring range. The given antigen content for each skin prick test solution is the mean value of four dilutions in range. The absorption values were calculated as ng/mL antigen on the basis of the protein concentration of a crude mould extract from Allergon as standard. For *A. alternata*, a monoclonal antibody-based sandwich ELISA kit against the major allergen Alt a 1 from Indoor technologies (Charlottesville, VA, USA) was used. For *A. fumigatus*, the Asp f 1-specific sandwich ELISA from Indoor technologies was performed, in addition to pAb sandwich ELISA, according to the manufacturer's instruction.

To quantify the total content of sIgE-binding mould allergens in the different skin prick test solutions, inhibition of sIgE binding was performed with ImmunoCAP system. Fifty microlitres of pooled serum (with a final sIgE concentration between 2 and 3 kU_A/L) were mixed with 10 µL of skin prick test solution and then incubated with the respective ImmunoCAP. As reference (100% binding and 0% inhibition), PBS instead of skin prick test solution was mixed with the serum pool and incubated on the respective ImmunoCAP. The percentage of reduction of sIgE binding by pre-incubation with the skin prick test solution was calculated compared with the PBS-reference and indicated as percentage of inhibition (% inhibition). The range of inhibition was defined between 20 and 80%; inhibition values < 20% were evaluated as no inhibition, whereas values > 80% inhibition were evaluated as complete inhibition.

In a second step, proteins from commercial mould SPTs were biotinylated, concentrated by speedvac centrifugation and loaded on Streptavidin-ImmunoCAPs (o221, Phadia, Uppsala, Sweden) as described by Sander et al. [31].

Statistical analysis

Linear regression, correlation (Pearson and Spearman) and two-tailed p-values of protein versus antigen versus allergen content of the mould skin prick test solutions were calculated with GraphPad Prism software (GraphPad Software Inc., CA, USA); *P*-values < 0.05 were considered as significant.

Results

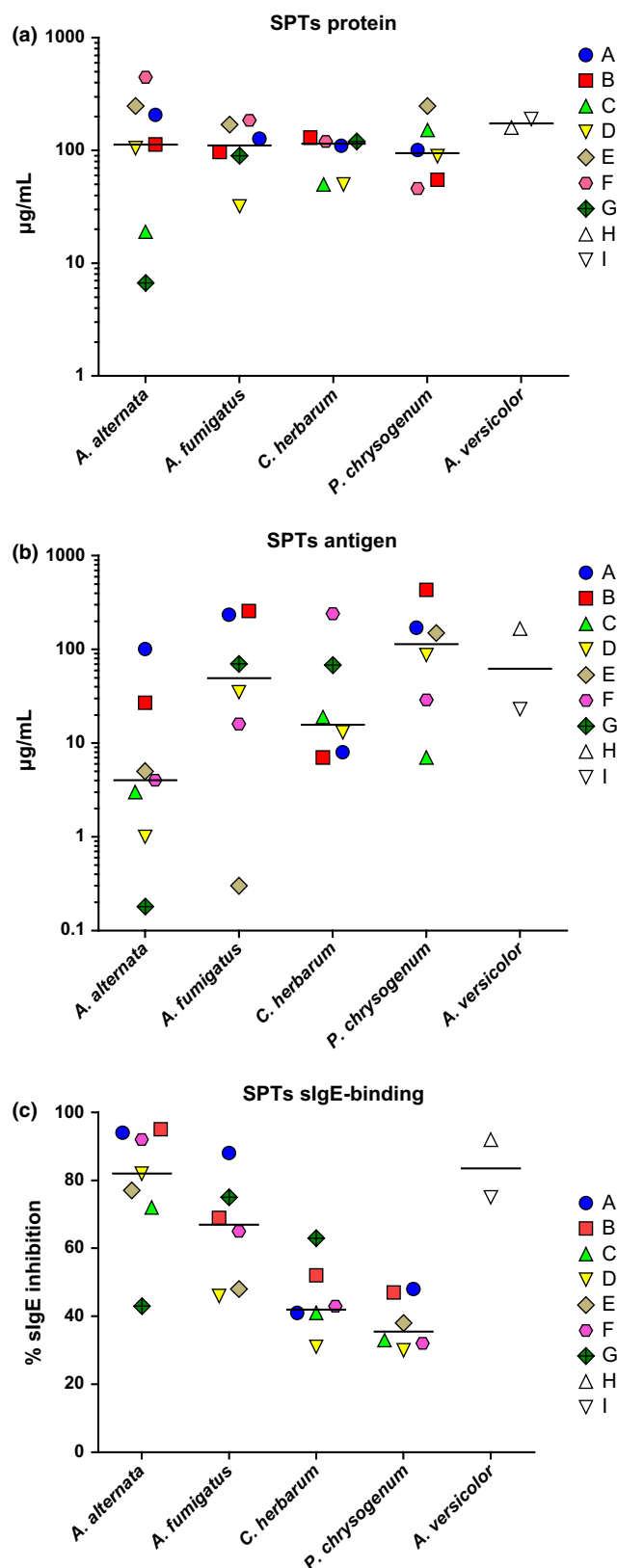
Quantitative analysis of protein, antigen and IgE-binding content in SPTs

Protein concentration of mould SPTs covered a range from below 10 µg/mL up to 447 µg/mL (Fig. 1a). The median protein value for all commercial mould extracts

was between 95 and 115 µg/mL. The median value for *A. versicolor* protein was 175 µg/mL. The considerable variability among protein concentration in SPTs was not assigned to special manufactures by means of a company internal protein content titration of SPTs (Table 2A). For example, protein content of mould SPT solution differed in company A between 101 µg/mL and 207 µg/mL, whereas in company G, the variability of protein content was between 7 µg/mL and 120 µg/mL. Protein content of SPTs was also not associated with a single mould species. For example, protein content in SPTs from *A. alternata* was not *per se* higher than any others.

Antigen concentrations measured by specific sandwich ELISA in mould skin prick test solutions were highly variable (Fig. 1b). The antigen contents of all SPTs ranged from 0.2 µg/mL to 430 µg/mL (Table 2B). In *A. alternata* SPTs, only the major allergen, Alt a 1 was measured covering a broad range from 0.18 to 101 µg/mL, with a median value of 4 µg/mL. The antigen concentration of *A. fumigatus* SPTs varied strongly, with a nearly 1000-fold difference between the lowest and highest values (range 0.3–256 µg/mL, median 53 µg/mL). The corresponding Asp f 1 concentration (Table 2B) was between 0.1 and 100 ng/mL with a median value of 8 ng/mL. Thus, Asp f 1 amount varied between 1/30 in SPTs from manufacturer D to 1/350 000 in SPTs from manufacturer B. Other SPT extracts exhibited variable antigen amounts (Fig. 1b and Table 2B) with median values of 16 µg/mL for *C. herbarum* (range: 7–240 µg/mL), 119 µg/mL for *P. chrysogenum* (range: 7–430 µg/mL) and 95 µg/mL for *A. versicolor* (range: 23–167 µg/mL). Antigen amount in all SPTs was independent of mould species and/or manufacturer.

Specific IgE-binding capacity of SPTs was analysed by sIgE-inhibition studies using corresponding ImmunoCAP as the solid allergen phase, SPTs as inhibitor and pooled human sera as sIgE source. All SPTs reduced the IgE binding to the corresponding ImmunoCAPs by more than 20%, proving that sIgE-binding components were present in all extracts (Fig. 1c). The median value of *A. alternata*'s SPTs showed a complete sIgE inhibition of 82%, indicating that all *A. alternata*'s SPTs, except that from manufacturer G (Table 2C), contained enough specific allergen to omit sIgE binding to ImmunoCAPs. Allergen extracts from *A. fumigatus* reduced sIgE binding in median by 67%. SPT solutions for *C. herbarum* and *P. chrysogenum* exhibited less IgE-binding components comparable with ImmunoCAP. In both cases, the medians of the sIgE inhibition were below 50% reduction (Table 2C). The two in-house produced SPT extracts for *A. versicolor* almost completely inhibited (75 and 92%) sIgE binding to solid-phase allergens (Table 2C).



Additionally, sIgE-inhibition tests were performed with SPTs on recombinant major allergens, rAlt a 1 and rAsp f 1, as well as with rAsp f 2, rAsp f 3, rAsp f 4

Fig. 1. (a–c) Quantitative protein (a), antigen (b) and allergen (c) content of 25 commercial SPTs from *Aspergillus alternata*, *A. fumigatus*, *Cladosporium herbarum* and *Penicillium chrysogenum* from different manufacturers (A – G), as well as two in-house extracts from *A. versicolor* (manufacturers H and I). Different skin prick test (SPT) solutions from manufacturers (A – I, see Table 1) were assigned to one specific icon, and mould species were indicated on the x-axis. Median values are shown as a line.

and rAsp f 6 as solid phase (ImmunoCAP, Table 2C). *Alternaria alternata* SPTs from manufacturers A and B reduced sIgE binding to recombinant Alt a 1 ImmunoCAP by more than 80%. *A. alternata* SPTs from manufacturers C–G inhibited IgE binding to rAlt a 1 about 50–60%. In the case of *A. fumigatus* single allergens as solid phase (Table 2C), only sIgE binding to Asp f 2 was reduced by about 43% with *A. fumigatus* SPT from manufacturer A and to about 22% by SPT from manufacturer C. IgE binding to rAsp f 1 could be inhibited by only one *A. fumigatus* SPTs (manufacturer A) to about 19%, which is in accordance with the defined inhibition range (20 – 80%) at the lower limit. All other recombinants Asp f 3, 4 and 6 were not inhibited by the applied *A. fumigatus* SPTs.

To verify the specificity of sIgE-inhibition tests, all mould SPT were coupled to ImmunoCAPs and sIgE-binding capacity was measured with the same pool serum as used for inhibition tests (Table 2D). *Alternaria alternata* SPT solutions of companies A and B reached with 8.68 and 8.35 kU/L about 80% sIgE-binding capacity referred to Phadia *A. alternata* ImmunoCAP, m6 (10.8 kU/L), but displayed the same CAP-class 3. Other SPT solutions of *A. alternata* from company C to F quantified pool serum as CAP-class 2 (compared with CAP-class 3 with m6). Only ImmunoCAP coupled with SPT solution of company G displayed sIgE value of CAP-class 0. These results of the *A. alternata* SPT extracts corresponded well with the sIgE-inhibition results. The correlation coefficient r (Spearman): 0.9286 and P -value: 0.0067 emphasized this significant relation.

For SPT solutions of *A. fumigatus*, *C. herbarum* and *P. chrysogenum*, measured sIgE values were remarkably lower (maximally 25 – 40% compared with the value obtained with the commercial Phadia ImmunoCAPs). Accordingly, the sIgE values received with the SPT ImmunoCAPs were about one CAP-class lower compared with the Phadia ImmunoCAP (Table 2D). *Aspergillus versicolor* was an exception; here, the prepared SPT solutions produced the same sIgE values of the pool serum as the commercial Gm25 ImmunoCAP. Correlating the SPTs sIgE values with the inhibition rates of each SPT solutions, the correlation coefficient of *A. fumigatus* was r (Spearman) = 0.7143, of *C. herbarum* r (Spearman) = 0.8407 and of *P. chrysogenum*: r (Spearman) = 0.7143. In sum-

Table 2. (A–D) Quantification of protein concentration (A), antigen concentration (B), IgE-binding potential in inhibition tests (C) and IgE binding to immobilized mould SPTs (D)

		Total protein content of SPT solutions (µg/mL)										
(A)	<i>Alt a</i>		<i>Asp f</i>			<i>Cla h</i>		<i>Pen ch</i>		<i>Asp v</i>		
Company A	207		127			110		101		–		
Company B	113		97			130		55		–		
Company C	19		–			50		152		–		
Company D	105		32			50		89		–		
Company E	248		170			–		248		–		
Company F	447		185			120		46		–		
Company G	7		90			120		–		–		
Company H	–		–			–		–		160		
Company I	–		–			–		–		190		
Antigen content of SPT solutions (µg/mL) assessed by ELISA ^(1–6)												
(B)	<i>Alt a</i> (1)		<i>Asp f</i> (2)		<i>Asp f</i> (3)		<i>Cla h</i> (4)		<i>Pen ch</i> (5)		<i>Asp v</i> (6)	
Company A	101		234		0.1		8		170		–	
Company B	27		256		0.001		7		430		–	
Company C	3		–		–		19		7		–	
Company D	1		35		0.0001		13		87		–	
Company E	5		0.3		0.01		–		150		–	
Company F	4		16		0.006		240		29		–	
Company G	0.2		70		0.013		68		–		–	
Company H	–		–		–		–		–		167	
Company I	–		–		–		–		–		23	
IgE inhibition test with SPT solution as inhibitor on ImmunoCAPs (% inhibition)												
		<i>Alt a</i>		<i>Asp f</i>						<i>Cla h</i>	<i>Pen ch</i>	<i>Asp v</i>
		m6	m229	m3	m218	m219	m220	m221	m222	m2	m1	Gm25
(C)	Alt a	rAlt a 1	Asp f	rAsp f 1	rAsp f 2	rAsp f 3	rAsp f 4	rAsp f 6	rAsp f 6	Cla h	Pen ch	Asp v
Company A	94	83	88	19	43	7	2	0	0	41	48	–
Company B	95	80	69	0	4	0	2	0	0	52	47	–
Company C	72	57	–	–	–	–	–	–	–	41	33	–
Company D	82	54	46	0	22	0	7	0	0	31	30	–
Company E	77	60	48	0	0	0	8	0	0	–	38	–
Company F	92	59	65	0	0	5	2	0	0	43	32	–
Company G	43	51	75	0	4	1	4	0	0	63	–	–
Company H	–	–	–	–	–	–	–	–	–	–	–	92
Company I	–	–	–	–	–	–	–	–	–	–	–	75
IgE to mould SPT solution coupled to streptavidin ImmunoCAPs												
		<i>Alt a</i>		<i>Asp f</i>		<i>Cla h</i>		<i>Pen ch</i>		<i>Asp v</i>		
(D)		(kU _A /L)	CAP-class	(kU _A /L)	CAP-class	(kU _A /L)	CAP-class	(kU _A /L)	CAP-class	(kU _A /L)	CAP-class	
Company A		8.68	3	4.84	3	1.53	2	0.73	2	–	–	
Company B		8.32	3	1.57	2	2.55	2	0.99	2	–	–	
Company C		1.36	2	–	–	1.12	2	0.22	0	–	–	
Company D		1.41	2	1.14	2	0.95	2	0.37	1	–	–	
Company E		1.31	2	0.18	0	–	–	0.41	1	–	–	
Company F		5.94	2	2.04	2	1.27	2	0.26	0	–	–	
Company G		0.30	0	1.22	2	1.58	2	–	–	–	–	
Company H		–	–	–	–	–	–	–	–	2.79	2	
Company I		–	–	–	–	–	–	–	–	2.48	2	
Phadia ImmunoCAP		10.8	3	12.3	3	10.8	3	2.57	2	2.53	2	

(1) and (3) ELISA based on mAb against Alt a 1 and Asp f 1 (Indoor Technologies, Charlottesville, USA); (2), (4), (5) and (6) ELISA based on pAb against *A. fumigatus* (2), *C. herbarum* (4), *P. chrysogenum* (5) and *A. versicolor* (6) crude extract described in [28, 30]

mary, the direct sIgE-binding values to immobilized proteins from SPT solution were comparable with sIgE-inhibition rates generated by competition of SPT solution with the commercially available solid-phase allergen on ImmunoCAPs.

Qualitative analysis of protein, antigen and IgE-binding components in mould SPTs

Mould SPT solutions were analysed for protein pattern, IgG-binding pattern (either mAb or pAb) and human IgE-binding pattern. An overview of all protein profiles of commercial SPTs for *A. alternata*, *A. fumigatus*, *C. herbarum* and *P. chrysogenum*, as well as for the two in-house SPTs of *A. versicolor* are shown in Fig. 2.

Analysis of A. alternata SPTs

Skin prick test solution of *A. alternata* from companies A and B showed comparable protein pattern (Fig. 2, *A. alternata*, SDS-PAGE silver stain lanes A and B) with major protein bands at a molecular weight of 14 kDa, corresponding to the molecular weight (MW) of Alt a 1. The 14 kDa band was also visible in all other preparations with differences in the intensity and number of bands. However, all bands were faint in SPTs from manufacturers C and G, correlating with low protein content. The SPTs from manufacturer D showed a faint 14 kDa band in comparison with other bands. In SPTs from manufacturer E, an additional band at 66 kDa was seen in all mould SPTs independent of the mould species, and SPTs from manufacturer F had high background. Corresponding IgG blots were performed with Alt a 1 specific mAb demonstrating detectable Alt a 1 in all *A. alternata* SPT solutions with a band at 14 kDa (Fig. 2, mAb/pAb IgG blot lanes A – G). In four SPTs, the dimer of Alt a 1 (30 kDa) was also detected (lane/manufacture A, B, E, F). Using the human IgE serum pool, Alt a 1 was detected only in SPTs from manufacturers A and B (Fig. 2, human IgE blot), which were analysed to have high amounts of Alt a 1 with mAb-sandwich ELISA (Table 2B).

Analysis of A. fumigatus SPTs

The protein pattern of *A. fumigatus* SPTs (Fig. 2, *A. fumigatus*, SDS-PAGE silver stain) from manufacturers A and B were again similar with prominent bands at 6, 19, 34 kDa, plus numerous protein bands between 35 and 95 kDa. Meanwhile, *A. fumigatus* SPTs from manufacturer D presented with a completely different image, with four distinct bands at 8, 16, 30 and 54 kDa. The protein pattern of *A. fumigatus* SPTs from manufacturer E had again the dominant 66 kDa band plus a faint single at 8 kDa; solution from manufacturer F

exhibited again strong background with several small protein bands; and the solution from manufacturer G had two distinct protein bands above 28 kDa. Even though the protein pattern of different *A. fumigatus* SPTs were heterogenic, corresponding IgG blots with *A. fumigatus*-specific pAbs (Fig. 2, *A. fumigatus*, mAb/pAb IgG blot) recognized similar antigens at molecular weights 16, 29, 32 and > 55 kDa in all SPTs but with different intensities. Human IgE-binding profiles (Fig. 2, *A. fumigatus*, human IgE blot) showed identical IgE-binding patterns for SPTs from manufacturers A and B. Human IgE binding to SPTs from manufacturer D was seen at 15 and 55 kDa. In SPTs from manufacturer E, no sIgE-binding proteins were detectable, whereas, SPTs from manufacturer F showed strong IgE binding at proteins of 80 kDa. IgE-binding proteins in SPTs from manufacturer G were again detected within the higher molecular weight range > 29 kDa. An assignment of protein bands to known *A. fumigatus* major allergens (www.allergen.org), such as Asp f 1 (18 kDa), Asp f 2 (37 kDa), Asp f 3 (19 kDa), Asp f 4 (30 kDa) or Asp f 6 (27 kDa) was difficult using SDS-PAGE or immunoblot.

Analysis of C. herbarum SPTs

Protein profiles of SPT solutions from *C. herbarum* were highly comparable between manufacturers A and B, with distinct protein bands at 16 and 20 kDa, plus several protein bands between 5 and 100 kDa. Skin prick test solution from manufacturers C, D and F showed faint bands between 8 and 20 kDa, whereas the protein pattern of SPT from manufacturer G had a major band at 16 kDa. All IgG-binding patterns did not show well-defined antigen bands, but showed strongest reactions to antigens > 29 kDa. In contrast, human IgE bound prominently to 24 kDa proteins in SPTs from manufacturers C and G. Known *C. herbarum* allergens (www.allergen.org) within this molecular weight range are Cla h 7 (22 kDa) and Cla h 8 (28 kDa).

Analysis of P. chrysogenum SPTs

Penicillium chrysogenum protein patterns (Fig. 2, *P. chrysogenum*, SDS-PAGE silver-stained) of SPTs from manufacturers A, B and D were comparable showing prominent double bands at 16–18 kDa, in contrast to SPTs from manufacturer C that exhibit 8 to 10 prominent bands from 8 to 80 kDa. In SPTs from manufacturers E and F, several faint bands were stained at MW below 16 kDa and above 40 kDa. The polyclonal antibody binding patterns of SPTs from manufacturers A–C were very similar even though the protein pattern from manufacturer C was strongly different in silver-stained SDS-PAGE (Fig. 2, *P. chrysogenum*, mAb/pAb IgG blot).

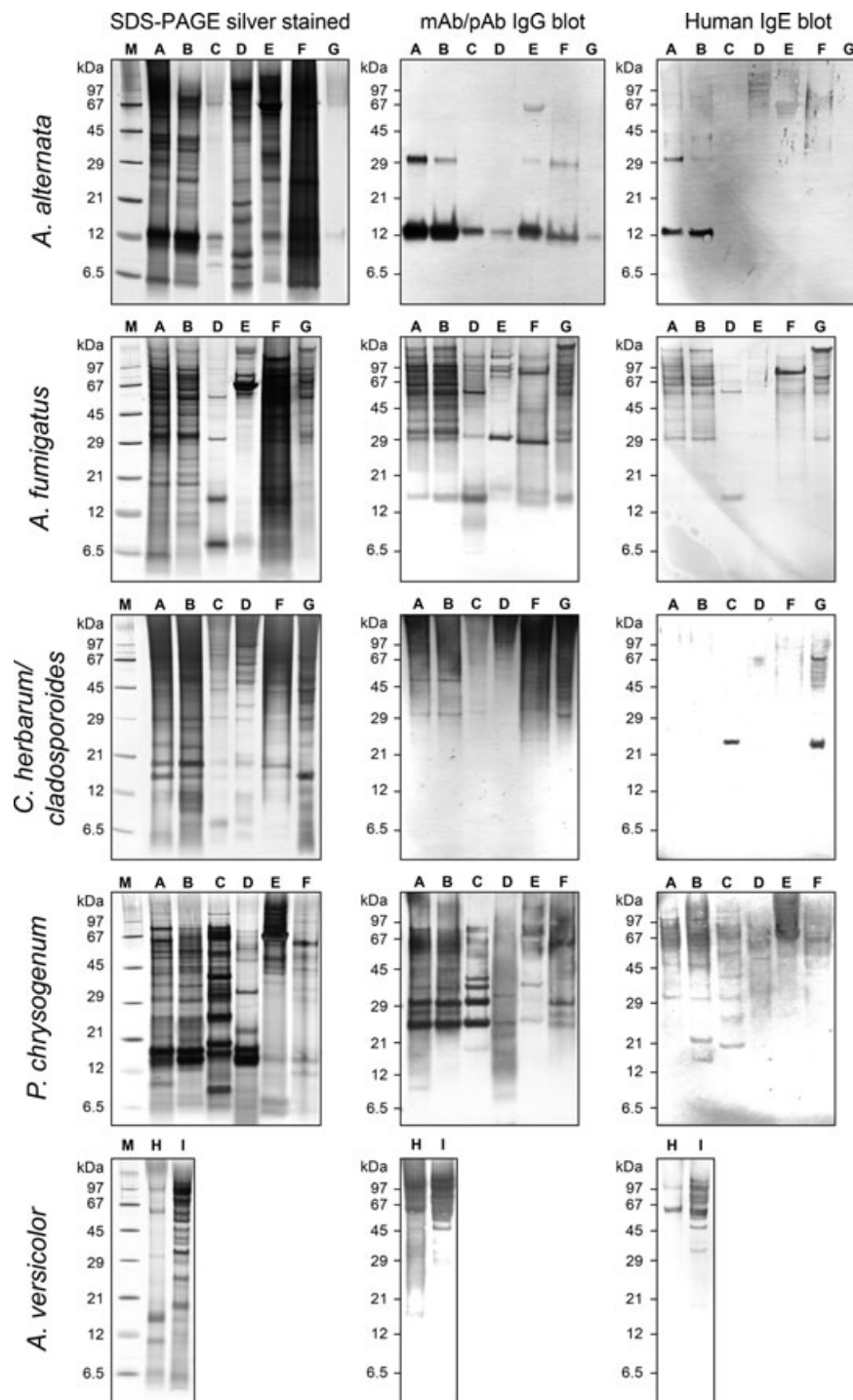


Fig. 2. SDS-PAGE and immunoblots of *Aspergillus alternata*, *A. fumigatus*, *Cladosporium herbarum* and *Penicillium chrysogenum* from commercial manufactures (see Table 1) as well as two *A. versicolor* in-house extracts. The first image is always a silver-stained protein profile of skin prick test solutions for one mould species supplied by different manufacturers (A – I). The second image shows the corresponding antigen image incubated with mould-specific mAb or pAb. The third picture is the sIgE-binding protein profile, detected by human IgE pooled sera.

Antigens were detected at 24, 30, 38 and 40 kDa. Antigen binding in SPTs from manufacturers D-F appeared more diffuse with defined signals above 24 kDa. Human IgE binding occurred as diffuse bind-

ing with clear signals at 14 and 22 kDa in SPTs from manufacturer B, and proteins of 21 and 26 kDa in SPTs from manufacturer C (Fig. 2, *P. chrysogenum*, human IgE blot).

Analysis of *A. versicolor* SPTs

Aspergillus versicolor SPT solutions were produced in parallel from two different source materials (Allergon (manufacture H) and Greer (manufacture I)) using the same extraction procedure. The obtained protein spectrum was clearly different between the two solutions (Fig. 2, *A. versicolor*, SDS-PAGE silver-stained). Skin prick test solution based on *A. versicolor* material from manufacture H showed four dominant protein bands at 7, 11, 15 and 60 kDa, in contrast to SPT solution prepared from *A. versicolor* material from manufacturer I. Here, up to twelve distinct protein bands were visible between 5 and 90 kDa. Antigens detected with the pAb were seen in the molecular weight range > 50 kDa (Fig. 2, *A. versicolor*, mAb/pAb IgG blot) and human IgE serum pool recognized a 60 kDa protein as the dominant allergen in both SPTs, and proteins at 35, 47, 65 and > 70 kDa in material from manufacturer I allergens. The only described allergen for *A. versicolor* so far is Asp v 13 at 43 kDa (www.allergen.org).

Is there any correlation of analysed parameters in SPTs?

Linear regression of the analysed parameters in all mould SPTs showed only a slight but significant correlation between protein concentration and sIgE inhibition with $r_{\text{(Spearman)}} = 0.4404$ and $P = 0.0215$. By calculating protein, antigen and sIgE-inhibition correlations for each mould species separately, it was seen that significant relations exist between protein amount and sIgE inhibition for *C. herbarum* SPTs ($r_{\text{(Spearman)}} = 0.8508$, $P = 0.033$), and between Alt a 1 concentration in SPTs and sIgE inhibition on *A. alternata* ImmunoCAPs (m6) with $r_{\text{(Spearman)}} = 0.7857$ and $P = 0.048$. For Alt a 1 concentration in SPTs and sIgE inhibition on Alt a 1 (m229) ImmunoCAPs, even an ideal correlation of $r_{\text{(Spearman)}} = 1$ and $P = 0.0004$ was calculated.

Discussion

In allergy, skin prick tests are an important diagnostic tool, because of their relative low costs, quick turnaround to results and minimal invasion for the patient. Standardized quality of allergen test extracts is a prerequisite for reliable test results [32]. Enhancing the quality and standardization of allergen extracts is an ongoing focus of many studies like CREATE [33]. In our study, the selected commercial mould SPT extracts analysed were highly variable in respect to their protein, antigen and allergen contents. The reason for this high variability within the SPTs of one mould species can be attributed to the different steps of the fungal extract production. First of all, the raw mould material is important. This is dependent on the choice of the specific

mould strain and the growth conditions, such as the culture medium, submerge or plated mould cultures, temperature, light, sporulation and duration of cultivation. The second step of mould SPT production - the extraction procedure - is also a source of variation. Extraction methods, extraction media and additives can influence the final protein, antigen and allergen content of the extract. Quality control, the final step in the SPT procedure, is another potential source of variability where the allergen quantification methods, such as mAb or pAb test systems compared with human IgE inhibition test systems, and the used standards (single allergens versus crude extracts) are likely sources. Accordingly, there are many possibilities to explain the variability among allergen extracts of the same mould species.

Even though the protein concentrations in most mould SPT solutions were similar, the protein patterns in SDS-PAGE showed clear differences, suggesting that measuring protein content alone is not a sufficient tool to determine quality of allergen extracts. The average protein content of 0.1 µg/µL in mould SPTs was lower compared with the described protein contents from other fungal allergen products from the US ranging from 0.25 to 0.73 µg/µL [19]. However, this variation could be attributed to a different protein measurement method or protein standard. The fact that protein patterns of mould species could be highly variable was previously described for *A. alternata* SPTs by Esch [22]. In this study, the variability seen with *A. alternata* was attributed to the more than 100 strains available from certified vendors of fungal material. Next to natural variations in protein patterns caused by different mould strains, non-mould proteins were also observed to be added to stabilize the SPT solution, such as the 66 kDa protein in all SPTs from manufacturer E.

Sensitive tools to quantify major allergen concentrations include sandwich ELISAs. In mould allergen extracts, two major allergens, Alt a 1 and Asp f 1, were quantified so far [22, 23, 34]. For all other mould species, no commercial antigen tests are available. The lowest Alt a 1 concentration measured here was 0.2 µg/mL, and the highest concentrated SPTs contained 101 µg/mL. These are relatively high levels of Alt a 1. In the study by Esch [22], seven *A. alternata* products from different manufacturers were quantified by ELISA inhibition and showed Alt a 1 concentrations between 0.04 and 2.75 µg/mL. In the study by Grier et al. [23], two *A. alternata* extracts were compared containing 10 – 40 µg/mL Alt a 1. Another study [34] quantified Alt a 1 in eight *A. alternata* products from US companies by sandwich ELISA ranging from 0.01 to 6.09 µg/mL. In all three studies, Alt a 1 levels were about 5- to 40-fold lower than in *A. alternata* SPT solutions (Alt a 1 content of 0.18 to 101 µg/mL) measured in our study. The differences in Alt a 1 content

may be caused either by methodical differences, such as the quantification test systems and Alt a 1 standards or by differences in the source material for *A. alternata*.

Another reason for strong variation is that Alt a 1 content depends on the cultivation conditions. Alt a 1 was shown to be a secreted protein that is expressed, in the late growth phase between 21 and 30 days as described by [35] and (www.allergon.com: poster EAACI2006). Therefore, the time of harvesting *A. alternata* material for allergen extract production is not the only important factor. It was further shown that Alt a 1 doubled at a growing temperature of 25°C compared with 20°C (www.allergon.com: poster EAACI2006), and cultivating the mould in suspension media increased Alt a 1 levels 4-fold compared with *A. alternata* grown on agar plates [35]. Ibarrola et al. [35] further showed that IgE-binding activity was 15-fold stronger in medium-cultured *A. alternata* material compared with agar-cultivated material. In this study, the secretion process of Alt a 1 was confirmed by immune electron microscopy. In young spores and hyphae, Alt a 1 was located in the cytoplasm, whereas in mature spores and hyphae, allergen labelling occurred exclusively in the cell wall and extracellularly.

The amount of Alt a 1 necessary to induce a skin reaction comparable to 1% histamine dihydrochloride solution was previously calculated [36]. The diagnostically relevant Alt a 1 concentration (quantified with a two-sided ELISA using Alt a 1-specific mAb and pAb, and purified Alt a 1 as standard) was 3.7 µg/mL. However, it must be considered that the Alt a 1 quantification test used in the latter study is different from the one used in our study, where four of the seven *A. alternata* SPTs were above 3.7 µg/mL. Two of three SPTs with Alt a 1 concentration below 3.7 µg/mL had low total protein content, whereas the third case had high total protein, despite the low Alt a 1 concentration.

Expression of Asp f 1 was shown to peak on day 3 of mould cultivation (in the early growth phase) [37]. Asp f 1, like Alt a 1, is a secretory protein predominantly found in culture medium filtrate; however, Asp f 1 content in spores and hyphae were very low. Arruda et al. postulated that germination of spores and fungal growth were necessary for Asp f 1 expression, which may explain the very high IgG and IgE levels in sera from patients with ABPA, aspergilloma and cystic fibrosis – all of whom have *A. fumigatus* in their lungs. The major allergen (Asp f 1) content was measured in eight *A. fumigatus* extracts from US manufacturers using mAb-sandwich ELISA [30]. The resulting concentrations (from less than 0.1 up to 64 µg/mL) was about 1000 times higher than what was measured in our study encompassing seven extracts from Europe. However, the values are difficult to compare because of the different test systems.

Biochemical analyses have revealed that SPT products are quantitatively and qualitatively highly heterogeneous, raising the question of comparability of SPT results. Subsequently, a multicenter study is conducted to test skin reactivity of different SPTs of *A. alternata*, *A. fumigatus*, *C. herbarum*, *P. chrysogenum* and two in-house extracts of *A. versicolor*, respectively.

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Conflict of interest

The authors declare no conflict of interest.

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