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Optimized methods for fungal α -amylase airborne exposure assessment in bakeries and mills

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Summary

Background In order to enable reproducible and comparable exposure measurements of fungal α -amylase (α -amylase) in different laboratories and countries, the entire procedure from sampling of airborne dust to measuring extracted samples (including standards and the used enzyme) immunoassays must be standardized. The aim of this study was to establish optimal elution and assay conditions.

Methods A parallel sampler was used for simultaneous collection of 10 samples of inhalable dust in bakeries and mills in Germany, England, the Netherlands and Spain. Three enzyme-immunoassays (EIAs) for detection of fungal α -amylase based on monoclonal antibodies or polyclonal antibodies were used for the measurement of the parallel-sampled filters ($n = 432$) extracted using several methods. The results were analysed by regression analysis of variance. Additional filters ($n = 54$) were extracted and analysed using two EIAs to investigate the storage stability of the extracts.

Results Although α -amylase concentrations correlated well ($r \geq 0.88$), differences were found between the EIAs in the sensitivity and nominal values (up to a mean factor 5.8). The best elution medium for airborne filters (phosphate-buffered saline 'PBS' with 0.05% Tween-20) led to 1.2 to two times higher α -amylase allergen yields than extraction in PBS only, while higher Tween-20 concentrations decreased the extracted α -amylase yield. During storage of frozen dust/filter extracts for 3–4 months at -20°C , a loss of approximately 40% of measurable α -amylase was observed, which could be partially prevented by addition of 0.1% casein to the medium directly after extraction.

Conclusion Although the effects of only a few of many possible causes of variation were investigated, for these factors a clear choice could be made with regard to optimal elution conditions and the use of validated EIAs with calibrated standards, thus making significant progress towards a completely standardized procedure for airborne α -amylase measurements.

Keywords α -amylase, Baker's asthma, exposure assessment, occupational allergy, standardization

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Introduction

Fungal α -amylase (α -amylase) is used frequently in bakeries and is a well-known occupational allergen, as shown by many case and population studies in Germany [1], France [2], Switzerland [3], Spain [4, 5], Sweden [6, 7], Belgium [8, 9], Italy [10], England [11, 12], Finland [13], the Netherlands [14] and Poland [15, 16]. The enzyme is

added to cereal flours to accelerate the baking process and improve the quality of bakery products. The amount needed is very small, and the final amylase content is estimated at only 0.4–10 mg/kg flour. Assays for monitoring fungal α -amylase at workplaces thus must be highly sensitive and specific. Using a two-site enzyme immunoassay (EIA) based on rabbit polyclonal antibodies (Pab), Houba and colleagues [14, 17, 18] and Nieuwenhuisen

et al. [12] demonstrated dose-response relationships between α -amylase exposure and allergen-specific sensitization, even at levels $<1 \text{ ng/m}^3$ as an 8-h time-weighted average. Other laboratories have developed EIAs based on monoclonal antibodies (Mab) for measuring fungal α -amylase [19, 20].

The results of allergen quantification, however, may depend on the methods used, e.g. on the binding characteristics and specificity of antibodies, the choice of the calibration standard or the media in which the allergens have been eluted from filters and stored until assay. Any comparison of exposure data from different countries and/or the definition of threshold limits for α -amylase exposure depend on the use of methods that can be reproduced in different laboratories. Two of the above-mentioned EIAs have been compared in a previous study [21]. Parallel aliquots of the same samples showed well-correlating results, but the measured concentrations showed up to threefold differences. Thus, to obtain accurate estimates of fungal α -amylase exposure, it is not enough to have very sensitive and specific methods but also to standardize the methods, such that either all laboratories would apply exactly the same procedures, or that well-established conversion factors be used for results from different centres.

The main objective of this study was to identify optimal conditions for the elution and routine EIA analysis of fungal α -amylase in airborne dust samples from the work environment. EIAs from three institutes were included: the Pab-based EIA of the Institute for Risk Assessment Sciences (IRAS, Utrecht, the Netherlands) [17], an adaptation of the Mab-based EIA of the Health and Safety Laboratory (HSL, Buxton, UK) [19] and the Mab-EIA of the Research Institute of Occupational Medicine of the Berufsgenossenschaften (BGFA, Bochum, Germany) [20]. The latter EIA was amplified following procedures for EIA amplification of the Karolinska Institutet (KI, Stockholm, Sweden) [22, 23]. These assays were used to analyse a series of airborne dust samples collected with a parallel sampler in bakeries and flour mills in the Netherlands, Germany, Spain and United Kingdom.

Methods

Airborne dust sampling for α -amylase measurements

Parallel dust sampling. A parallel sampler [24], enabling simultaneous collection of 10 samples of inhalable flour dust with 10 PAS-6 sampling heads and a flow rate of 2 L/min/head, was used to collect samples for comparisons of elution procedures in bakeries and flour mills in the Netherlands (NL), Germany (DE), Spain (ES) and United Kingdom (UK), at eight locations (two locations per country). All sampling was performed with coded polytetrafluoroethylene (PTFE) filters with 1 μm pore size

(FALP2500, Millipore, Billerica, MA, USA) and nearly 10% efficiency of particle retention that had been pre-weighed at IRAS (Utrecht, NL) and then distributed to the various participating institutes. A total of 800 filters were collected (200 per country) and in each country from at least two workplaces at which parallel samples were taken on 10 occasions, during which the sampling time varied from 30 min to 6 h to obtain a range of dust and allergen levels. This parallel sampling series was the same as that used for wheat allergen test methods by Bogdanovic et al. [24]. After sampling, filters were returned to IRAS for post weighing, while being kept at ambient temperature and low humidity in air-tight containers (Petrilide, Millipore) during transport and storage. For the extraction experiment, nine of the 10 filters of six runs from each worksite (108 filters per country) were initially selected for post weighing and further analysis.

Because of the low numbers of amylase-positive samples, it was decided to improve the statistical power of the study by extracting another 54 filters from six parallel sampling runs obtained from worksites where relatively high amylase concentrations were observed.

Extraction experiments

Extraction of filters was performed at IRAS. Briefly, four major extraction parameters were investigated: choice of extraction fluid, elution tube, shaking method and centrifugation speed (see Table 1). Each parameter had three different options, which were combined (two parameters with three options/run, 3×3 , thus nine filters/run) for the extraction of initially 432 filters (48 runs), using per extraction day two options as a fixed parameter while two other parameters were varied. After extraction, 2 mL of the eluates were divided into nine portions (8×0.2 and $1 \times 0.4 \text{ mL/tube}$), and frozen at -20°C . Of each eluate, three aliquots were shipped on dry ice to both BGFA and HSL for amylase measurement by EIA. After analysis of the first 432 samples, 54 additional filters from locations with relatively high amylase concentrations were extracted while varying only the extraction fluid and shaking method.

Determination of α -amylase concentrations in extracts

The α -amylase allergen levels were determined by three enzyme immunoassays: the amplified version of the BGFA Mab EIA, the IRAS Pab EIA [17] and a Mab EIA [19] developed and performed at the Health and Safety Laboratory, Buxton, UK. Initially, 432 extracts were measured. Sample dilutions were initially 1/2–1/4–1/8 in all assays, and re-testing at higher dilutions was performed if values at 1/8 dilution exceeded the upper detection limits of the assay. The additional series of 54 samples was only tested in the IRAS and the BGFA assay. If a first test failed

Table 1. Investigated extraction parameters

Extraction parameters	Investigated options	No. of extracted filters
Elution medium	PBS, pH 7.4, volume = 2.5 mL	72+18
	PBS Tween-20 0.05% (v/v), pH 7.4, volume = 2.5 mL	288+18
	PBS Tween-20 0.5% (v/v), pH 7.4, volume = 2.5 mL	72+18
Tube	Minisorp Nunc, 4 mL	72
	PolyStyrene round bottom, 4 mL	288+54
	Glass culture tube, 4 mL	72
Shaking method	End-over-end-roller, 60 min	288+18
	Horizontal shaker, 60 min	72+18
	Intermittent manual vortexing for 5–10 s (beginning, after 30 and 60 min)	72+18
Centrifugation speed	0 g = no centrifugation	72
	1000 g, 15 min	72+54
	3000 g, 15 min	288

PBS, phosphate-buffered saline.

for technical reasons, the test was repeated with another fresh aliquot. In total, valid data were produced for all 486 extracts analysed at IRAS and BGFA, and for 391 of 432 tested extracts at HSL.

Pab-based enzyme-immunoassays for fungal α -amylase (IRAS). The assay was performed using the sandwich EIA protocol by Houba et al. [17], with slight modifications. Microtitre plates were coated overnight at 4 °C with affinity-purified anti-amylase Pab (2 µg/mL in PBS), and free-coating sites were blocked with PBS+Tween-20 (0.05% v/v) containing 0.2% (w/v) gelatine (PBTG). Test samples and serial dilutions of the amylase standard (Fungamyl 1600S, Novo, Denmark) were added in PBTG and incubated for 1 h at 37 °C, after which bound amylase was detected and measured by subsequent incubations with biotinylated immunospecific anti-amylase IgG (1/500 in PBTG), avidin-peroxidase (1/2000 in PBTG; Dako, Glostrup, Denmark) and, as a substrate, 0.011 M *o*-phenylenediamine (OPD; Sigma, Taufkirchen, Germany) with H₂O₂ in phosphate-citrate buffer. After stopping with 2 N HCl and reading optical density (OD) at 492 nm, the concentration of sample dilutions was calculated from a log-log regression line of the standard, using Softmax Pro 2.6.1 from Molecular Devices (Sunnyvale, CA, USA). The lower level of detection (LOD) was the concentration corresponding to an OD₄₉₂ = 0.05 above the background value. The α -amylase concentration of the calibration standard was defined as the protein concentration of the solution measured by Lowry analysis with bovine serum albumin (BSA) as the reference material [17].

Amplified Mab-based enzyme-immunoassays for fungal α -amylase (KI-BGFA). Two different anti-fungal α -amylase Mabs ('Anton' IX 3c4, 'Berta' III 1b4) were combined in a sandwich-EIA procedure based on the original BGFA EIA [20], with amplification steps developed by Karolins-

ka Institutet [22, 23]. Ninety-six-well plates (Maxisorp, Nunc, Denmark) were coated with Mab 'Anton' (~8.3 µg/mL in carbonate/bicarbonate buffer, pH 9.6) and blocked with casein buffer (PHRPDILX, RDI, USA). Serial dilutions of α -amylase standard (Fungamyl 800L, Sigma A8220, Taufkirchen, Germany) and samples in casein buffer were then incubated, and amylase binding was detected with biotinylated affinity-purified anti-amylase Mab 'Berta' (~0.67 µg/mL), followed by polymeric peroxidase-conjugated streptavidin (SA-poly-HRP80, RDI, diluted 1 : 20 000 in casein buffer) and finally enhanced K-blue[®] substrate solution (Neogen, Lexington, KY, USA). The enzyme reaction was stopped after 10 min with 1 M H₂SO₄ and the absorbance was read at 450 nm and 650 nm. Sample concentrations were read by interpolation of OD values (OD_{450 nm} – OD_{650 nm}) on a four-parameter dose-response curve of the standard preparation, using Softmax Pro 4.7.1 from Molecular Devices. The LOD was the concentration corresponding to OD = 0.05 above the estimated minimum value ('parameter A') of the 4-parameter curve fit function.

The α -amylase content of the standard in this assay (Fungamyl 800L) was defined as its protein concentration, as determined by a Bradford assay, with BSA as the reference protein.

Mab-based enzyme-immunoassays for amylase (Health and Safety Laboratory). This method was an adaptation of a fungal α -amylase protocol described previously [19] using newly prepared primary and secondary antibodies from an alternative clone source. This was necessary due to the dwindling stocks of original Mabs and clones. The assay was automated for use on the Rosys Plato robotic system (Robotec, UK). Microwells were coated with monoclonal anti-amylase antibody HSL-2D2 at 5 µg/mL (IgG from high-titre mouse anti-fungal- α -amylase antiserum). Diluted test samples and α -amylase standard (Megazyme,

Ireland) were incubated in PBS – 0.1% Tween – 1% BSA and amylase binding was detected with a 1/1500 dilution of biotinylated anti-amylase Mab HSL5C7 (IgG from high-titre mouse anti-FAA antiserum), followed by a 1/3000 dilution of horseradish streptavidin (Vector Labs, Peterborough, UK) and finally 3,3',5,5'-tetramethylbenzidine (TMB) (SureBlue), KPL (Gaithersburg, MD, USA). The plates were read at 450, and the data were processed using the Rosys Plato onboard software. The limit of detection was defined as the lowest concentration that could be differentiated from zero with 95% certainty (this was derived from an immunoassay precision profile program, ProQuant (QIVX Inc., Fort Collins, CO, USA). It was noted that the limit of detection using the alternative Mabs was considerably higher and that the assay overall was found to be less sensitive than the original EIA developed by HSL. The α -amylase content of the standard was defined as its protein concentration as determined using the bicinchoninic acid protein assay (BSA as a reference standard).

Extract storage experiment. The additional set of parallel filters ($n = 54$) was also used to assess the effect of extract storage at -20°C , and the modifying effects of adding casein to the medium. After extraction (three different buffers, three shaking options), each eluate was split into two 1 mL portions, of which one was mixed with 0.1 mL of a 1% casein solution. Both portions were then divided into small aliquots, and stored at -20°C . A first EIA test was performed after 4 days (KI/BGFA Mab EIA) or 2 weeks (IRAS Pab-EIA) with aliquots of both casein-containing and casein-free extracts, and 3 months later the tests were repeated with freshly thawed aliquots.

Data analysis

Because only 5–30% of the extracts (depending on the EIA) showed detectable α -amylase allergen levels, mixed regression analysis of variance (ANOVA, mixed linear models using the procedure PROC MIXED in the statistical software package SAS 8.2, SAS, Cary, NC, USA) was carried out with three sets of data: (1) all values included, with non-detectable allergen levels replaced by 2/3 of the lower LOD; (2) only results from runs with at least four values within the run above the detection limit in EIAs; and (3) only results from runs with at least four values above the detection limit in EIAs, and within which extractions varied in a specific extraction parameter.

The choice of 'run' and not 'extract' as the unit of inclusion (or exclusion) was based on the design of the extraction experiments, in which parameters were systematically varied within each run. Less efficient elution procedures might easily lead to values $<\text{LOD}$ in a run with other samples from the same run having $>\text{LOD}$ values. Selection of positive extracts, and thus inclusion of only the latter in a data set used to assess the effect of

elution procedures would thus lead to serious artefacts. Because of the relatively low numbers of positive extracts, the data analysis was sensitive to bias if either too many samples with negative values were included (thus making differences more difficult to detect), or when too many negative values would be omitted.

All analyses were performed with natural log-transformed values, and the results were expressed as factors with confidence intervals, indicating a relative increase or decrease in yield compared with one of the (three) options of the extraction parameters set at 1.0, being the reference parameter. In all models, 'country' (where the samples had been collected) was included as a potential confounder, because the location where a certain run was performed was a strong determinant of the measured allergen levels.

Results

Comparison of enzyme-immunoassays in the parallel sampling experiment

Replicate aliquots of 486 filter extracts from the parallel sampling of airborne dust in bakeries and flour mills were analysed for their fungal α -amylase content at BGFA with the KI/BGFA-amplified Mab-based EIA, and at IRAS with the Pab-based EIA. Additionally, 391 of these extracts were also analysed at HSL Buxton, UK, using the HSL Mab EIA. Table 2 shows the distribution of positive samples at each of the different sampling locations, and for each of the various assays. Out of 486 filter extracts, 133 extracts showed detectable levels in the KI/BGFA Mab-EIA, and 147 in the IRAS Pab-EIA. Using the HSL Mab-EIA, only 18 out of 391 were detectable. The concordance and correlation between the assays (Fig. 1) were the best between IRAS and KI/BGFA EIAs (87% concordance, Pearson's and Spearman's correlation coefficients of 0.963 and 0.943, respectively, for log-transformed values of positive samples). HSL and KI/BGFA EIAs had a concordance of 85%, with Pearson's and Spearman's correlation coefficients of 0.935 and 0.944, and between HSL and IRAS EIAs a concordance of 82% with Pearson's and Spearman's correlation coefficients of 0.879 and 0.850 were obtained. The skewed distribution in the two comparisons with HSL data (Figs 1b and c) clearly illustrates the (much) lower sensitivity of the HSL assay. While the mean LOD of the HSL assay (using the newer but less sensitive Mabs) was 0.51 ng/mL Megazym α -amylase, the mean LOD of the BGFA/KI assay was 19 pg/mL Sigma α -amylase, and the mean LOD of the IRAS assay was 80 pg/mL Fungamyl 1600S.

The highest absolute α -amylase values were obtained with the assay from IRAS, followed by the values from HSL and BGFA. The geometric mean ratios of double-positive values for IRAS/BGFA, IRAS/HSL and HSL/BGFA

Table 2. Numbers and percentages of positive samples from different sampling locations (DE1 and DE2: small traditional bakeries in Germany, ES1: small traditional bakery, ES2: industrial bakery in Spain, NL1: flour producing company, NL2: industrial bakery in the Netherlands, UK1 and UK2: industrial bakeries in England) analysed using the three EIAs for fungal α -amylase

Location	IRAS Pab-EIA			KI/BGFA amp Mab-EIA			HSL Mab-EIA		
	N	N _{pos}	% _{pos}	N	N _{pos}	% _{pos}	N	N _{pos}	% _{pos}
DE1	90	64	71.1	90	66	73.3	54	17	31.5
DE2	54	4	7.4	54	2	3.7	51	1	2.0
ES1	54	15	27.8	54	20	37.0	38	–	–
ES2	72	33	45.8	72	20	27.8	54	–	–
NL1	54	9	16.7	54	6	11.1	38	–	–
NL2	54	20	37.0	54	17	31.5	54	–	–
UK1	54	2	3.7	54	1	1.9	54	–	–
UK2	54	–	–	54	1	1.9	54	–	–
Total	486	147	30.2	486	133	27.4	397	18	4.5

IRAS, Institute for Risk Assessment Sciences; Pab, polyclonal antibodies; EIA, enzyme-immunoassay; KI, Karolinska Institutet; BGFA, Berufsgenossenschaftliches Forschungsinstitut für Arbeitsmedizin; Mab, monoclonal antibodies; HSL, Health and Safety Laboratory.

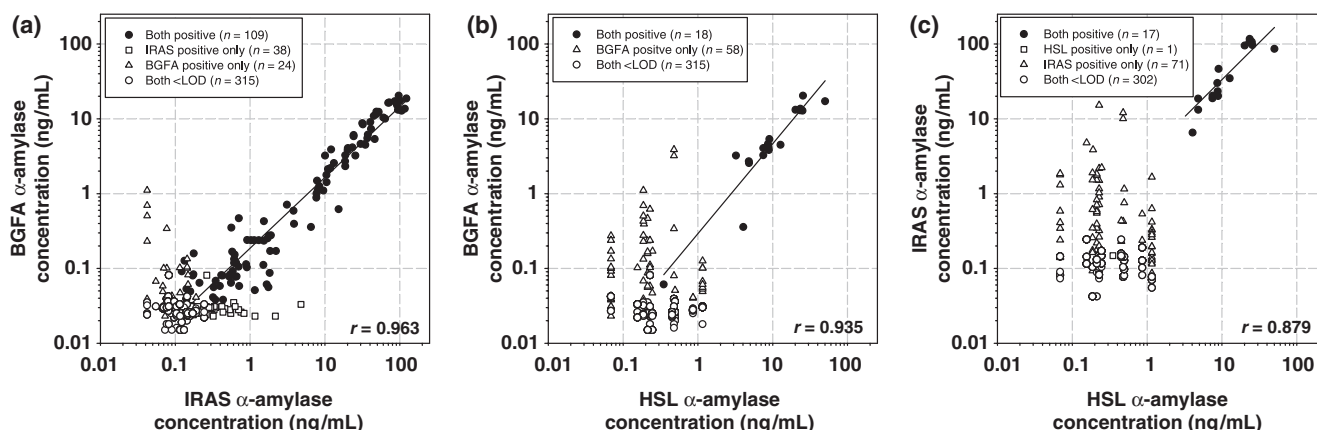


Fig. 1. Comparison of alpha-amylase (α -amylase) levels in extracts of airborne dust samples, as obtained by (a) BGFA assay and IRAS assay ($n = 486$), (b) BGFA assay and HSL assay ($n = 391$) and (c) IRAS assay and HSL assay. Values above detection limit in both compared assays (filled symbols) are shown together with the regression line and Pearson's correlation coefficient. IRAS, Institute for Risk Assessment Sciences; BGFA, Berufsgenossenschaftliches Forschungsinstitut für Arbeitsmedizin; HSL, Health and Safety Laboratory; LOD, level of detection.

EIAs were 5.8, 3.3 and 2.2, respectively. The highest measured fungal α -amylase exposure in the study was 780 ng allergen/m³ in the IRAS EIA, and the corresponding values in the two other assays were 260 ng/m³ (HSL) and 130 ng/m³ (KI/BGFA).

Analysis of the three different α -amylase standard preparations using the KI/BGFA Mab EIA assay revealed significant differences in specific activity, which appeared to be the highest for the Sigma preparation, 3.5-fold higher compared with the Fungamyl 1600s preparation, and 2.2-fold higher compared with the Megazyme amylase (data not shown).

Comparison of different extraction methods

The effects of variation in extraction parameters on the measured α -amylase levels were studied by mixed ANOVA. The results of the first model are shown in Table 3a. This

model, with all values included, showed for two of the three assays a significant (20–25%) higher allergen yield after extraction in PBS with 0.05% Tween-20 compared with PBS with 0.5% Tween-20. The second ANOVA model, with all data from runs with at least four positive filter extracts, was only run with the results of the IRAS and KI/BGFA EIAs (Table 3b) because of the very low numbers of positive results in the HSL EIA (Table 2). In this model, a significant effect of extraction buffer was also found, with up to 1.7- to 2-fold higher yields in PBS with 0.05% Tween-20 than in PBS with 0.5% Tween-20. PBS alone showed a (non-significant, 25–50%) higher yield than PBS with 0.5% Tween-20. Among shaking procedures, the 'intermittent vortexing' method appeared to result in the highest allergen concentrations significantly, with approximately 40% lower values after extraction using the end-over-end roller. An almost twofold higher yield after centrifugation at 3000 g compared with no

Table 3. ANOVA results for four different extraction parameters, as obtained with: (a) statistical Model 1 (all data included) and three enzyme immunoassays (EIAs) for fungal α -amylase (IRAS, KI/BGFA, and HSL). (b) Model 2 (only data from runs with at least four positive samples) and two EIAs (IRAS and KI/BGFA). (c) Model 3 (I–IV) in which only data obtained from the IRAS and KI/BGFA EIAs were included, from runs with at least four positive samples, and in which each subset (I–IV) a specific single extraction parameter varied

	IRAS EIA (N= 486)		KI/BGFA EIA (N= 486)		HSL EIA (N= 391)	
Effect	Factor	CI	Factor	CI	Factor	CI
(a) Model 1						
PBS	1.05	(0.87–1.28)	0.96	(0.81–1.12)	1.16 [†]	(1.01–1.33)
PBS+0.05% Tween	1.26 [†]	(1.04–1.52)	1.08	(0.92–1.27)	1.20 [†]	(1.05–1.37)
PBS+0.5% Tween*	1		1		1	
End-over-end rotator	0.86	(0.71–1.03)	0.88	(0.75–1.04)	1.03	(0.90–1.18)
Laboratory shaker	0.89	(0.74–1.08)	0.88	(0.75–1.04)	0.93	(0.83–1.09)
Intermittent vortexing*	1		1		1	
Glass	0.88	(0.71–1.08)	0.94	(0.79–1.13)	0.96	(0.83–1.10)
Minisorp	0.94	(0.76–1.16)	1.11	(0.92–1.32)	1.01	(0.88–1.16)
Polystyrene*	1		1		1	
0 g	0.89	(0.71–1.10)	1.09	(0.84–1.21)	1.01	(0.88–1.16)
1000 g	1.10	(0.89–1.36)	1.01	(0.85–1.21)	0.99	(0.86–1.15)
3000 g*	1		1		1	
	IRAS EIA (N= 117)		KI/BGFA EIA (N= 117)			
Effect	Factor	CI	Factor	CI		
(b) Model 2						
PBS	1.52 [†]	(0.94–2.44)	1.26			(0.82–1.96)
PBS+0.05% Tween	1.96 [†]	(1.22–3.14)	1.67 [†]			(1.08–2.58)
PBS+0.5% Tween*	1		1			
End-over-end rotator	0.60 [†]	(0.38–0.94)	0.65 [†]			(0.43–0.98)
Laboratory shaker	0.70	(0.44–1.09)	0.76			(0.50–1.15)
Intermittent vortexing*	1		1			
Glass	0.97	(0.50–1.88)	0.81			(0.44–1.49)
Minisorp	1.06	(0.54–2.05)	1.03			(0.56–1.89)
Polystyrene*	1		1			
0 g	0.54 [†]	(0.30–0.98)	0.87			(0.50–1.50)
1000 g	0.82	(0.46–1.46)	0.74			(0.43–1.26)
3000 g*	1		1			
	IRAS EIA (N= 72)		KI/BGFA EIA (N= 72)			
Effect	Factor	CI	Factor	CI		
(c) Model 3						
(I)						
PBS	1.52	(0.91–2.51)	1.26			(0.81–1.97)
PBS+0.05% Tween	2.00 [†]	(1.20–3.32)	1.69 [†]			(1.08–2.63)
PBS+0.5% Tween*	1		1			
	IRAS EIA (N= 81)		KI/BGFA EIA (N= 81)			
Effect	Factor	CI	Factor	CI		
(II)						
End-over-end rotator	0.63 [†]	(0.43–0.91)	0.68 [†]			(0.48–0.94)
Laboratory shaker	0.70 [‡]	(0.48–1.01)	0.76			(0.54–1.06)
Intermittent vortexing*	1		1			
	IRAS EIA (N= 36)		KI/BGFA EIA (N= 36)			
Effect	Factor	CI	Factor	CI		
(III)						
Glass	1.11	(0.48–2.55)	0.89			(0.40–1.97)
Minisorp	1.20	(0.52–2.77)	1.13			(0.51–2.50)
Polystyrene*	1		1			

Table 3. continued

Effect	IRAS EIA (N = 45)		KI/BGFA EIA (N = 45)	
	Factor	CI	Factor	CI
(IV)				
0 g	0.52 [†]	(0.32–0.84)	0.83	(0.50–1.38)
1000 g	0.73	(0.45–1.19)	0.67	(0.40–1.12)
3000 g*	1		1	

ANOVA outcomes are given as factors indicating the estimated mean relative recovery of allergen, with 95% confidence intervals (CI), compared with the referent option/setting/value of the parameters (*).

[†]Significantly different from the referent parameter, $0.001 < P < 0.05$.

[‡]On the borderline of statistical significance, $0.05 < P < 0.1$.

IRAS, Institute for Risk Assessment Sciences; PBS, phosphate-buffered saline; EIA, enzyme-immunoassay; KI, Karolinska Institutet; BGFA, Berufsgenossenschaftliches Forschungsinstitut für Arbeitsmedizin; Mab, monoclonal antibodies; HSL, Health and Safety Laboratory.

centrifugation at all was obtained using the IRAS assay, but not with the BGFA assay.

The third ANOVA approach focused on separate analysis of the effects of a single extraction parameter. In each analysis, it included only runs with at least four positive samples, and for which the parameter of interest had varied during extraction. This approach had been chosen in order to achieve a maximal 'signal-to-noise' ratio in analysis, because runs with variation in outcome due to other factors were excluded. Results as summarized in Tables 3c (I–IV) largely confirmed the findings of the previous models, with the highest yields of measurable amylase allergen after extraction with PBS+0.05% Tween-20 and much lower concentrations found with PBS+0.5% Tween-20. Up to 30–50% higher yields were noted after intermittent vortexing, compared with other shaking methods, and the highest yields after centrifugation at 3000 g. No effect from the extraction vial was found in these analyses.

Extract storage

When measurements of filter extracts were repeated, 66.7% (30 of 45) of the samples with detectable levels in the first test showed lower values in the second test. These results suggested a loss of activity in filter extracts over time. To explore this further, the extracts of the additional set of 54 parallel filters were stored frozen in the absence or presence of added casein (0.1%, w/v), and all were tested twice using the KI/BGFA and IRAS EIAs: shortly after extraction and after a few months of storage at -20°C . With both EIAs, a loss of α -amylase reactivity was found after extract storage for 3 months, as shown in Fig. 2a for the KI/BGFA. This loss could be partially avoided by adding casein (Fig. 2b). The effect of casein was analysed in more detail, differentiating between

the different extraction buffers with a mixed ANOVA, and using the following fixed effects in the model: buffer $\times$ storage $\times$ casein, shake method, country, and the run number as a random effect. The results showed that, after 3 months of storage, there was indeed a loss of activity, especially if the extracts contained 0.5% Tween-20 and no casein (50–60%). With the optimal Tween-20 concentration (0.05%), the loss of activity was less ($\sim 30\%$), and in the case of storage with casein for 3 months and measurement by KI/BGFA assay, it was only 10% (data not shown).

Discussion

In most studies on exposure measurements of fungal α -amylase so far, [8, 12, 14, 18, 25] the Pab-based immunoassay developed by Houba et al. [17] has been used. Airborne bakery dust was sampled on PTFE filters in all these studies, but elution of filters was performed with different buffers and detergent concentrations (without Tween-20 [8, 14, 18, 25], or after addition of 0.5% v/v Tween-20 [12]). In other studies, the immunoassays based on Mab were used for detection of α -amylase [19, 20] in extracts of airborne dust sampled on glass fibre filters [19, 26, 27]. For reliable α -amylase allergen assessment, it is important to improve, compare and evaluate these measurement methods, and select the optimal ones for future use by all research groups. In this study, we aimed to optimize elution and EIA methods for fungal α -amylase, using airborne dust samples from the bakery and flour mill work environment in four European countries. To obtain replicate filters with the same dust and allergen load for comparison of extraction methods, parallel samplers with 10 sampling heads loaded with PTFE filters were used. Owing to the study size, the potential effects of filter type on allergen yields could not be explored. PTFE filters were chosen for their smooth filter surface, which is

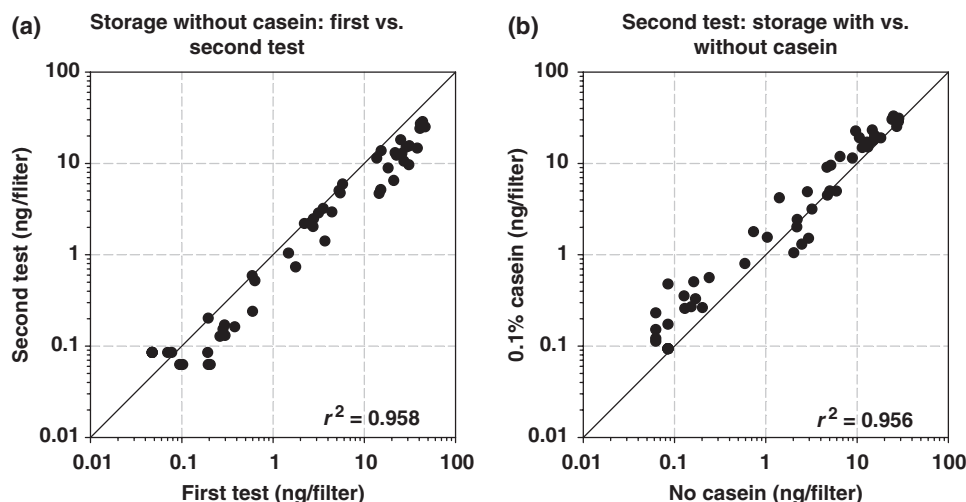


Fig. 2. Correlation of amylase values ($n = 54$) measured by the KI/BGFA immunoassay (a) after 4 days (first test) and 3 months (second test) and (b) after 3 months with and without addition of casein. In each plot the line of identical values is shown. BGFA, Berufsgenossenschaftliches Forschungsinstitut für Arbeitsmedizin; KI, Karolinska Institutet.

presumed to minimize absorption and loss of protein, and because they absorb very little of the added extraction fluid. In contrast to, for example, the highly porous glass fibre filters, nearly the whole volume of the medium used for the elution of PTFE filters can be recovered and used for the different allergen assays.

Three different immunoassays were compared – one using polyclonal (IRAS) and two using Mab (KI/BGFA [20, 23], and HSL [19]). The comparisons showed a high correlation between results (Pearson's correlation coefficients ≥ 0.88). The sensitivity of the assays was, however, quite different: out of 391 filter extracts, 88 (23%) and 76 (19%) were positive in the IRAS and KI/BGFA EIAs, and only 18 (4.6%) in the HSL EIA. The generally low number of positive samples is not an artefact found only in this study, but has been noted in most previous α -amylase exposure studies [8, 12, 14, 27].

Significant differences in absolute values were also observed: the IRAS EIA consistently showed the highest and the KI/BGFA EIA the lowest values, with a factor 5.8 difference between these two assays. The difference was mainly due (factor 3.5) to the use of different standards for the assays, and also the different methods to define the protein content of the standard (Lowry vs. Bradford). Between the IRAS and the HSL EIA, a factor of 3.3 was found, half of it (factor 1.5) explained by the observed difference in the activities of the standards used. The KI/BGFA and HSL EIA differed by a factor 2.2, which was the same as the difference between the standards.

Thus, both the choice of the standard and the method for defining its α -amylase allergen/protein content greatly influenced nominal values and should be clearly defined for all α -amylase EIAs. As the production of Fungamyl 1600S (the preparation used as the α -amylase

standard in nearly all former studies) has stopped, the authors recommend Fungamyl 800L as a common standard for all future α -amylase assays, with a common method for estimation of the protein content. This method could be amino acid composition analysis, which is also recommended for EIAs used for allergen standardization in diagnostics and immunotherapy [28].

To find optimal elution conditions, four extraction parameters were varied: elution medium, shaking method, type of vial and centrifugation speed. The outcome of the extraction experiments showed some heterogeneity depending on the selected data set on which the ANOVA was performed, and on the EIA results used as an outcome parameter. Nevertheless, the combined results provide sufficient evidence for several important recommendations. While addition of a small amount of the detergent Tween-20 increased the α -amylase yield, a significant loss of measured allergen content was observed with all three EIAs after extraction with a high concentration (0.5%) of Tween-20. This effect is unique for fungal α -amylase, as no adverse effects of high Tween-20 concentrations have been reported for other allergens [24, 29, 30].

Measurements of extracts after several months of storage at -20°C showed significant losses of α -amylase concentrations that were most pronounced in the presence of 0.5% Tween in the buffer. Thus, a high Tween-20 concentration may have affected the conformation of α -amylase such that it became more susceptible to proteolytic degradation. The loss of α -amylase during storage could be partially prevented by the addition of casein. Therefore, PBS with 0.05% Tween-20 is recommended as the extraction buffer giving the highest allergen yields, and addition of 0.1% casein is recommended for storage. This extraction and storage medium has also been shown

to function well for wheat allergens [24], which are often measured in the same sample. For storage of extracts before α -amylase quantification, it is thus recommended to add 0.1% casein to the buffer directly after extraction before freezing and to measure amylase allergen by EIA within one, and certainly no more than 2–3 months of storage at -20°C . Further experiments may be needed to assess whether other storage temperatures (-80°C , or even in liquid nitrogen) might allow long-term storage without any losses.

Other extraction parameters had much less influence on allergen yields. The shaking method using 'intermittent vortexing' (three times 5–10 s vortexing, separated by two 30-min intervals during which tubes are simply left to stand) can be recommended as a simple and convenient procedure that gave on average higher allergen yields than the other two shaking procedures. The type of extraction vial used had little impact. The basic and inexpensive (polystyrene) plastic tube can therefore be recommended. Centrifugation at 3000 g after extraction gave on average higher amylase yields than no centrifugation at all or centrifugation at 1000 g.

In conclusion, optimal elution conditions and highly sensitive EIAs are very important for measurement of airborne α -amylase exposure, as airborne fungal α -amylase concentrations – even at 'high' exposure sites or during 'high exposure' tasks – are in fact very low with maximal fungal α -amylase exposure values between 130 and 780 ng/m³ in this study, and amounts captured on filters are often near or below the EIAs detection limits. Short-term sampling (30–60 min), as partially performed during this study, did not capture enough allergen for detection.

Furthermore, airborne levels of α -amylase at a worksite (including those measured on filters from the parallel sampler) showed large variations in time and space. Thus, for reliable personal exposure assessment multiple measurements would be required, e.g. repeated full-shift (ca. 8 h) or half-shift (4 h) measurements.

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