



Comparison of Four Methods to Assess Fungal α -amylase in Flour Dust

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The aim of the study was to compare four different immunological methods to analyse fungal α -amylase in flour dust samples. Three European research groups have independently developed four immuno assay based methods to measure α -amylase in air samples. Three of the methods use polyclonal antibodies and one method uses monoclonal antibodies. Eighty personal samples have been collected during two to three work-shifts in four bakeries. Sampling was performed with PAS-6 inhalable dust samplers and aliquots from each sample were analysed by the three laboratories. The agreement between the four methods was high compared with agreement between immunological methods to measure other allergens in the air, e.g. for rat allergens. For the three methods with polyclonal antibodies the mean differences for individual samples was less than a factor of two. The arithmetic means (AM) of the estimated α -amylase exposure were 12.5, 11.3, 8.6 and 25.9 ng/m³ for the respective methods with values ranging from below the detection limit to 192, 215, 207 and 615 ng/m³. The AM for all samples analysed by the methods with polyclonal antibodies varied by about a factor of 1.5. About one-third of the values were below or at the detection limit for all methods. In a regression analysis the squared correlation coefficients (R^2) between the methods varied between 0.91 and 0.95 for the log transformed values. For workplace monitoring, results from the methods using polyclonal antibodies will be relatively comparable. High levels of α -amylase might differ in absolute numbers with a factor of two or more between the different methods but will anyway be considered as high and should result in preventive actions. On the other hand, this study also shows that despite the relative agreement between methods, there is a clear need for standardization. © 2000 British Occupational Hygiene Society. Published by Elsevier Science Ltd. All rights reserved.

Keywords: fungal α -amylase; flour dust; immunological methods; monoclonal antibodies and polyclonal antibodies

INTRODUCTION

Since the 1980s the use of fungal α -amylase as a

dough improver has increased in many bakeries. α -amylase is a powerful sensitizing enzyme. Two studies (Houba *et al.*, 1996a; Nieuwenhuijsen *et al.*, 1999) have shown a strong and positive association between allergen exposure levels and α -amylase sensitization in the nanogram per m³ range. During recent years sensitive and specific immunological

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Table 1. Essential features and differences between the methods developed by the three laboratories to measure amylase in flour dust samples

	Laboratory			
	UW	S	BRD-p	BRD-m
Immunoassay	Sandwich enzyme immunoassay (EIA)	Sandwich EIA	Two-site polyclonal-antibody-based ELISA	Two-site monoclonal-antibody-based ELISA
Trapping antibody on microtitre plates	Anti amylase antibodies at 2.35 µg/ml in PBS	Anti amylase antibodies at 5 µg/ml in 50 mM carbonate buffer	Anti amylase antibodies at 7.5 µg protein/ml carbonate/bicarbonate buffer (pH 9.6)	Anti amylase antibodies at 7.5 µg protein/ml carbonate/bicarbonate buffer (pH 9.6)
Blocking of free coating sites	PBS-Tween containing 0.2% (w/v) gelatine (PBTG) for 30 min	1% BSA in PBS for 60 min	2% (w/v) BSA in PBS for 2 h	2% (w/v) BSA in PBS for 2 h
Standard solution	47–2000 pg/ml in PBTG	75–9600 pg/ml	50–20 000 pg/ml in PBS	50–20 000 pg/ml in PBS
Standard and sample incubation	Biotinylated amylase antibodies in avidin-peroxidase solution. As substrate solution <i>o</i> -phenylenediamine was used at 2 mg/ml in 0.05 mol/l phosphate buffer with 0.015% H ₂ O ₂	Biotinylated amylase antibodies in streptavidin-peroxidase solution. As substrate solution <i>o</i> -phenylenediamine was used according to the manufacturer	Biotinylated amylase antibodies and streptavidin-peroxidase. The assay was developed with 100 µl <i>o</i> -phenylenediamine activated with H ₂ O ₂ according to the manufacturer	Biotinylated amylase antibodies and streptavidin-peroxidase. The assay was developed with 100 µl <i>o</i> -phenylenediamine activated with H ₂ O ₂ according to the manufacturer
Stopping solution	50 µl/well of 2 N HCl	50 µl/well of 1 M H ₂ SO ₄	100 µl/well of 1 M H ₂ SO ₄	100 µl/well of 2 mM NaN ₃
Optical density (OD)	492 nm	492 nm	492 nm	450 nm
LOD (pg/ml)	100	65	150	600

methods to analyse α -amylase have been developed. The reported results in amylase levels might differ because of differences in methodology. The objective of this study was to compare four methods to analyse α -amylase in flour dust samples developed by three European research groups, i.e. University Wageningen (UW), Sahlgrenska University Hospital, Göteborg (S) and Ruhr-University, Bochum (BRD). The groups from The Netherlands and Sweden have developed methods using polyclonal antibodies from immunized rabbits and the German group has developed methods using either polyclonal or monoclonal antibodies (BRD-p and BRD-m). The work is a part of the concerted action programme 'Epidemiology of occupational allergic asthma and exposure to bioaerosols'.

MATERIAL AND METHODS

Study design

In total 80 representative samples were collected during two to three shifts in four bakeries in three countries: 29 samples in Sweden, 29 in Germany and 22 in The Netherlands. All samples were personal samples taken during a complete shift. In bakeries different task groups are involved in bread and pastry production, e.g. dough makers, bread-formers, confectionery workers, oven workers and packers. Workers wearing the sampling equipment were selected from different task groups to ensure different levels of amylase in the samples. The same person could wear sampling equipment from one shift up to three shifts. Sampling was performed with the PAS-6 inhalable dust sampler with 25 mm (i.d.) Teflon filters (PTFE) and the flow rate was 2 l/min. The same industrial hygienist performed the sampling in all three countries.

After sampling all filters were stored at -20°C until extraction. α -amylase on the filters was extracted at room temperature with 2.5 ml 0.15 M phosphate buffered saline (PBS, pH 7.4) in a 10 ml centrifuge tube in one of the participating laboratories (UW). Each tube was vortexed for 2 min, sonicated for 2 min, vortexed for 5 min and sonicated for 2 min successively. The extract was centrifuged at 5000 g for 15 min and the supernatant was stored at -20°C . Aliquots from each sample were distributed to the laboratories at Wageningen, Sahlgrenska University Hospital and Ruhr-University and were analysed within 9 months.

ANALYTICAL METHODS

The four methods to analyse α -amylase developed by the three research groups are presented in Table 1 (Houba *et al.*, 1996b; Sander *et al.*, 1997, 2000). For each analysis 100 μl of the dust sample extract was added undiluted per well. In

Wageningen and at Sahlgrenska Hospital a standard solution of α -amylase allergen was prepared by using lyophilized Fungamyl 1600S powder from NOVO (Denmark). The α -amylase was dissolved in phosphate buffered saline (PBS) and filtered. In Bochum purified α -amylase (Baur *et al.*, 1994) was used as the standard. Protein content of the amylase preparation was measured separately by the three groups.

STATISTICAL ANALYSIS

The detection limits (LOD) of the polyclonal antibody-based assays were 65, 100 and 150 pg/ml, respectively, for the methods in Göteborg, Wageningen and Bochum. For the method based on monoclonal antibodies used in Bochum the detection limit was higher, 600 pg/ml. For reported values below the detection limit the respective detection limit divided by the square root of two has been used. The detection limits in ng/m^3 were about 0.3, 0.2, 0.4 and 1.6, respectively, for whole shift dust samples.

The statistical analyses were performed using SAS software, version 6, and Excel 97. The amylase values analysed by the four methods were approximately log-normally distributed (w : normal = 0.83, 0.77, 0.82 and 0.78) for the methods in Wageningen, Göteborg and Bochum (polyclonal and monoclonal). The high number of values below the detection limit (36–45%) could partly explain deviation from the normal distribution.

The level of agreement between the methods is described by use of regression lines with X - Y plots and by calculating the geometric mean and the ratio between the levels reported for each sample and its confidence interval (Bland and Altman, 1986; Hollander *et al.*, 1999).

RESULTS

Descriptive statistics

Table 2 shows the arithmetic mean (AM), geometric mean (GM), geometric standard deviation (GSD) and ranges of α -amylase in ng/m^3 as found by the four methods. The arithmetic means of α -amylase exposure for the total number of samples were 12.5, 11.3, 8.6 and 25.9 ng/m^3 with α -amylase levels ranging from below the detection limit to 192, 215, 207 and 615 ng/m^3 , respectively. Arithmetic means varied by about a factor of 1.5 if the means of all samples using polyclonal antibodies were compared. The method with monoclonal antibodies used by Bochum (BRD-m) showed two to three times higher AM values.

Geometric means found in Wageningen (UW) and in Bochum with polyclonal antibodies (BRD-p) showed very similar values, when all samples were

Table 2. Means, GSDs and ranges of α -amylase levels in ng/m³ in inhalable dust assessed by the three laboratories in total samples and separated in samples taken in the three countries

Method/lab	Country	N	N < LOD	AM	Range	GM	GSD
UW	All samples	80	29	12.51	0.19–192.44	1.46	8
S	All samples	80	35	11.31	0.12–214.83	0.81	9
BRD-p	All samples	80	30	8.62	0.28–207.03	1.54	6
BRD-m	All samples	80	36	25.91	1.09–614.63	4.70	5
UW	Sweden	29	12	15.30	0.19–192.44	1.67	9
S	Sweden	29	13	10.38	0.12–170.41	0.78	8
BRD-p	Sweden	29	9	7.18	0.28–53.38	1.77	5
BRD-m	Sweden	29	15	22.00	1.09–237.56	4.74	6
UW	Germany	29	13	9.51	0.19–127.30	0.96	7
S	Germany	29	16	11.09	0.12–214.83	0.59	9
BRD-p	Germany	29	16	10.56	0.28–207.03	0.98	5
BRD-m	Germany	29	12	30.05	1.09–614.63	3.79	6
UW	Netherlands	22	4	12.80	0.19–77.52	2.17	8
S	Netherlands	22	6	12.82	0.12–117.56	1.28	10
BRD-p	Netherlands	22	5	7.95	0.28–36.46	2.33	5
BRD-m	Netherlands	22	9	25.65	1.09–165.47	6.18	6

compared, as well as if the samples from the three different countries were compared separately (Table 2). The GM values found in Göteborg (S) were about half the respective values in UW and BRD-p. The method using monoclonal antibodies (BRD-m) showed GM values three to six times higher compared with the methods with polyclonal antibodies. The α -amylase levels analysed with the different methods did not differ substantially in the three different countries.

Level of agreement

The regression line equations and squared correlation coefficients (R^2) for the comparison of the different combinations of the four methods using log-transformed values are shown in Table 3. In Fig. 1 and 2 two of these correlations are presented as X - Y plots. As a comparison the plot for identical values ($X = Y$) is also presented. The squared correlation coefficients varied between 0.91 and 0.95 and the regression lines varied from parallel to crossing the $X = Y$ line. The respective R^2 coefficients for the untransformed values were 0.82, 0.57, 0.65, 0.79, 0.88 and 0.97. The regression line between UW and S for untransformed values gave an equation almost identical with the $X = Y$ plot (Fig. 3). Theoretically, the high correlations could be the result of high numbers of samples below the detection limit. However, removal of samples with a

value below the detection limit hardly influenced the correlations between the different methods.

The calculation of the geometric mean and the relative difference (ratio) for each sample (two methods at a time) showed that UW values were as a mean 1.8 ($n = 80$, 95% CI 0.6–5.9) times the S values. At the same time the UW values were as a mean 0.95 ($n = 80$, 95% CI 0.3–2.7) and 0.3 ($n = 80$, 90% CI 0.8–1.3) times the BRD-p and BRD-m values. The BRD-p method showed 1.9 ($n = 80$, 95% CI 0.5–7.3) times the S method while the BRD-m method showed a mean of 5.9 ($n = 80$, 95% CI 1.4–25.3) times the S method and 3.1 ($n = 80$, 95% CI 1.1–8.7) times the BRD-p method.

The 12 blank samples were all below the detection limit for the respective methods.

DISCUSSION

The agreement between the four methods is quite high compared with the differences of several orders of magnitude found between a radioallergosorbent (RAST) inhibition method and a monoclonal enzyme linked immunosorbent assay for rat allergens (Renström *et al.*, 1997; Hollander *et al.*, 1999). The monoclonal antibody assay showed as a mean three to six times higher α -amylase values and a remarkably higher detection limit compared with the methods with polyclonal antibody assays.

Table 3. Regression equations and squared correlation coefficients for the different combinations of correlations

Combination of correlations	Regression equations	Squared correlation coefficient (R^2)
UW-S	$Y = 1.02X - 0.76$	0.93
UW-BRD-p	$Y = 0.82X - 1.34$	0.95
UW-BRD-m	$Y = 0.75X - 2.96$	0.91
S-BRD-p	$Y = 0.77X - 2.20$	0.94
S-BRD-m	$Y = 0.72X - 3.62$	0.94
BRD-p-BRD-m	$Y = 0.90X - 1.86$	0.91

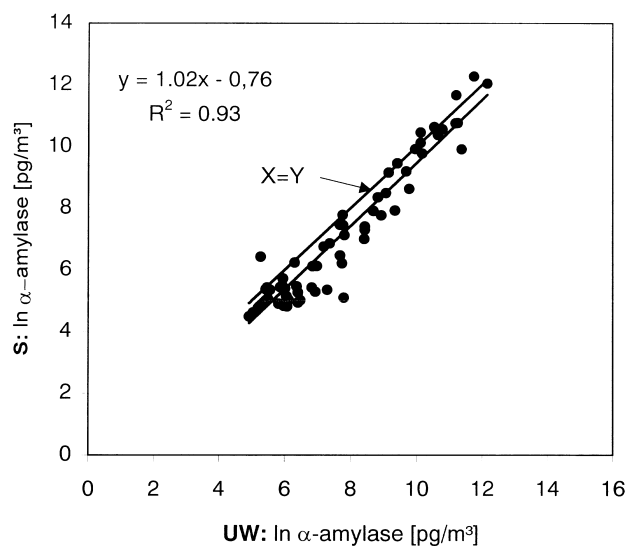


Fig. 1. Regression analysis (X - Y plots) of α -amylase levels analysed by UW and S methods. In the analysis the values in pg/m^3 are log-transformed and as a comparison the plot for identical values ($X = Y$) is also presented. α -Amylase samples were analysed by University Wageningen (UW) and the Sahlgrenska University (S).

Theoretically one would expect lower values when a monoclonal antibody is being used compared with polyclonal antibodies, since polyclonals are expected to detect more proteins present in a dust sample. One reason for the high values could be that purified α -amylase was used as a standard. During purification (ultrafiltration, ion-exchange chromatography, dialysis, and lyophilization) some denaturation, removal of epitopes, or aggregation of α -amylase might have occurred. Detection of the monoclonal antibody assay is based on two epi-

topes only. If denaturation changes one of these epitopes, the assay will not be able to measure α -amylase. If one assumes that the monoclonal antibodies detect only some of the α -amylase present in the standard, the amount of α -amylase in the samples will be overestimated. As a result, the detection limit of the monoclonal antibody-based assay will also be higher.

The BRD assay using polyclonal antibodies showed the lowest AM for all samples and also for samples from each country. Geometric mean values

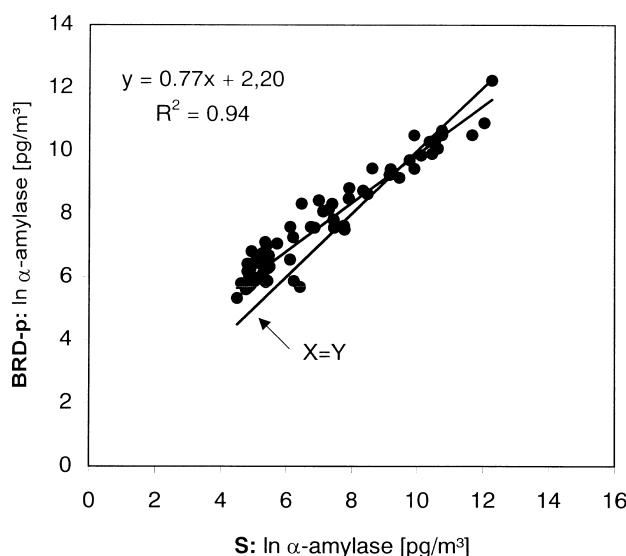


Fig. 2. Regression analysis (X - Y plots) of α -amylase levels analysed by S and BRD-p methods. In the analysis the values in pg/m^3 are log-transformed and as a comparison the plot for identical values ($X = Y$) is also presented. α -Amylase samples were analysed by Sahlgrenska University (S) and Ruhr-University in Bochum (BRD).

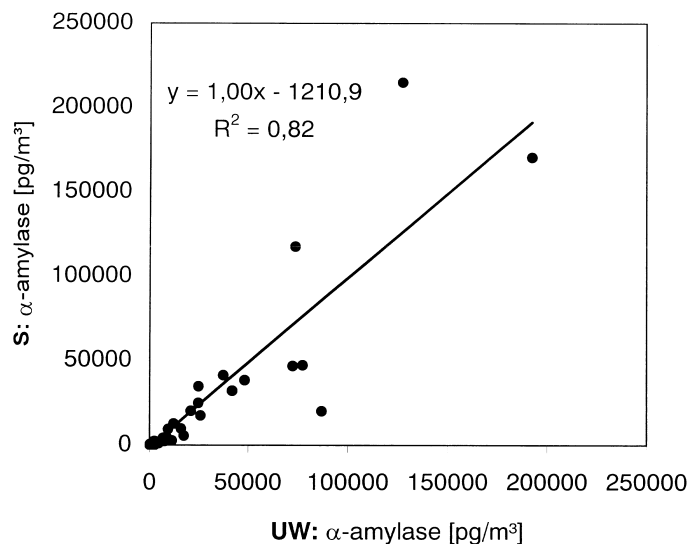


Fig. 3. Regression line for untransformed values of α -amylase levels in pg/m^3 analysed by the University Wageningen (UW) and Sahlgrenska University (S).

for the BRD-p method showed values very close to the GM values for the UW method, which indicates that there are some systematic errors between the methods in the analyses of samples with high or/and low content of α -amylase rather than differences in the standards used by the research groups.

Out of the 80 samples analysed for α -amylase content, 18 were below or close to the detection limits for all four methods, while with each method there were between 29 and 36 samples below the detection limit for the respective method. Still there was a high correlation between all four methods with R^2 values between 0.91 and 0.95 for the log-transformed values.

In one of the studies from the UK (Nieuwenhuijsen *et al.*, 1999) a strong and positive association was shown between α -amylase exposure and sensitization. In their study, dust samples were analysed using the UW method. Results from this study can therefore directly be compared with the exposure response study by Houba *et al.* (1996a), apart from differences in extraction methods and inhalable dust sampling heads used. The levels in the UK study were presented as mean values of 0.7, 10.7 and $46.7 \text{ ng}/\text{m}^3$, respectively. In another study (Burstyn *et al.*, 1998) mean levels of $22 \text{ ng}/\text{m}^3$ of α -amylase were reported, ranging from below the detection limit to $307 \text{ ng}/\text{m}^3$. In the discussion on hygienic limit value, values in the low ng/m^3 are considered (Heederik *et al.*, 1999), and it can be anticipated that the available evidence at least suggests that the hygienic limit value should be well below $50 \text{ ng}/\text{m}^3$, even in the absence of further harmonized methods to measure α -amylase in dust samples. However, more comparable exposure assessment

methods and assays would allow a more detailed evaluation of a health-based exposure standard and required exposure compliance strategies.

There was no systematic change in α -amylase content of the dust, if samples were reanalysed after freezing and thawing. Standard samples with low protein content were more sensitive to freezing and thawing.

In conclusion, the agreement between all four methods to analyse α -amylase is high compared with other immunological methods published recently for rat urinary proteins (Renström *et al.*, 1997; Hollander *et al.*, 1999). On the other hand, differences are to some extent artificially small compared with real life situations since, apart from the assays and the standards used, other factors might differ between laboratories as well. Filter media, extraction buffers, etc. are known to contribute to potential differences between methods to evaluate allergens and other bio-aerosols levels in airborne dust (Douwes *et al.*, 1995; Zock *et al.*, 1996).

For the three methods with polyclonal antibodies the differences in mean values were less than a factor of two. Thus, workplace monitoring with the different methods using polyclonal antibodies will give comparable results. The monoclonal antibody-based assay showed three to six times higher mean values compared with the other methods and the reasons should be further investigated. This study shows that despite the relatively small differences between laboratories for this type of assay, there is a clear need for standardization. Criteria for standardization need to be developed since some aspects are clearly different compared with methods to measure chemical agents in the work environment.

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