

LC-MS/MS quantification of airborne fungal α -amylase at a production facility

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ABSTRACT

Work-related exposure to bio-allergens, such as fungal α -amylase, is a well-known cause of occupational rhinitis and asthma. Therefore, it is important to have suitable analytical methods available so that exposure reduction measures can be taken when levels are too high. In this study, concentrations of airborne fungal α -amylase down to 5 ng/filter were determined by means of a new analytical method that is based on enzymatic digestion, using trypsin, followed by liquid chromatography (LC) and high-resolution accurate-mass (HR-AM) mass spectrometry (MS) with targeted selection of enzyme fragment ions of interest (tSIM). Quantification was performed using an external calibration curve based on a fungal α -amylase from *Aspergillus niger* (Fungamyl Ultra[®]). The manufacture of this enzyme and the acquisition of the analytical samples by air sampling and subsequent collection on PTFE filters took place at the same time at the same enzyme production facility. By means of the new HR-AM tSIM LC-MS method, the magnitude of occupational exposure can be elucidated.

KEYWORDS: airborne enzyme, HPLC, mass spectrometry.

INTRODUCTION

Fungal α -amylase (UniProt: P0C1B3) is the best characterized fungal enzyme found in the

occupational environment. Occupational sensitization to α -amylase was first reported by Flindt [1] and this enzyme has subsequently been identified as an allergen in the baking [2], pharmaceutical [3], animal feed [4], and biotechnology [5] industries. Exposure reduction measures based on systematic allergen monitoring can help to prevent or reduce the incidence of work-related allergies. Allergen measurement commonly includes airborne dust sampling at a workplace, and protein extraction and an allergen-specific assay at a laboratory. Among these assays, enzyme immunoassays (EIAs) have been reported for the detection of fungal α -amylase in extracts of personal dust samples [6, 7]. However, EIAs are labor-intensive, require specialized equipment and laboratory facilities and, including air sampling and sample pretreatment, take at least several days. To overcome these drawbacks, the specific antibodies developed for these EIAs were used to set up a simple one-step immunochromatography semi-quantitative assay for fungal α -amylase, based on the principle of a lateral flow immunoassay (LFIA) [8]. A comparative study of the LFIA and EIA for fungal α -amylase has been reported [9]. Non-immunological assays, such as LC-MS, have been published for the measurement of proteinaceous compounds [10, 11] and metaproteomic analysis [12] in atmospheric aerosol samples; however, to our knowledge, no non-immunological method has been described in the literature for the quantitative measurement of airborne fungal α -amylase. In this paper, we present a new LC-MS/MS method for the quantification of fungal α -amylase in airborne samples.

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MATERIALS AND METHODS

Reagents

Bovine serum albumin (BSA; P0914 and A3059), dithiothreitol (DTT; D0632), iodoacetamide (IAA; I1149), ammonium bicarbonate (A6141) and formic acid (1.00264) were supplied by Sigma Aldrich (The Netherlands). Trypsin from porcine pancreas modified by reductive methylation to reduce autolysis (V5280) was supplied by Promega Benelux (The Netherlands). LC-MS-grade water with 0.1% formic acid (84867.320E) and acetonitrile with 0.1% formic acid (84866.320E) were procured from VWR International (The Netherlands). Fungal α -amylase standard (FUNGAMYL ULTRA[®], batch AF307177, 650 mg/g) was obtained from DSM Food Specialties (The Netherlands). The amount of α -amylase in this standard was calculated based on a measured fungal amylase activity (FAU) and the known specific activity.

Aerosol sampling and protein extraction

Aerosol samples were collected at different places within an enzyme production facility. Approximately 0.2 to 0.4 m³ of air was sampled with calibrated GilAir Plus air sampling pumps (Sensidyne, St. Petersburg, FL, USA) at a flow rate of approximately 1.7 L/min and collected using PTFE filters (Millipore, FALP03700), this corresponding to the total time of the formulation process. As shown in Figure 1, the PTFE filters were fixed in a filter cassette during sampling. After sampling, these cassettes were sent in a closed bag at room temperature to DSM Biotechnology Center (Delft, The Netherlands) for analysis and stored at room temperature until analysis. Each cassette was sent separately to prevent cross-contamination. For protein extraction, 3 mL of buffer (50 mM ammonium bicarbonate) containing 8 μ g/mL BSA was added to each cassette. The cassettes were closed and mixed thoroughly on a vortex mixer for 5 min. BSA was introduced to reduce fungal α -amylase loss due to adsorption on the cassette or filter, and to eliminate the influence of varying protein concentrations on tryptic digestion. The total amount of BSA added to the filters was chosen in such a way that it provided a constant protein background enabling controlled sample preparation, having a fixed ratio of substrate protein (BSA + amylase) and trypsin of approximately

20:1, as recommended by the trypsin supplier. Approximately 1.5 mL of buffer was removed from the top of the filter cassette and 1 mL of this solution was transferred into an Eppendorf Protein LoBind tube. The remaining buffer solution was absorbed by the back filter. The concentration of the solution in the back filter was checked by the same method as for the top of the filter cassette.

The protein solutions (1 mL) extracted from the filter samples were dried until dryness at 40 °C by SpeedVac concentration (centrifugation and vacuum (0.1 bar)). Quantification of fungal α -amylase was performed using an external calibration curve based on Fungamyl Ultra[®] with a fungal α -amylase content of 650 mg/g. Calibration solutions of concentrations 0, 5, 10, 15, 20, 40 and 100 ng/mL were prepared in 50 mM ammonium bicarbonate buffer and 1 mL of these solutions and 2 mL of buffer (50 mM ammonium bicarbonate) were poured over blank filters, which were then processed using the same procedure as described for the aerosol samples. The dried proteins from both the filter samples and the calibration solutions were re-dissolved in 330 μ L of 100 mM ammonium bicarbonate buffer. 200 μ L of this solution was used for tryptic digestion and LC-MS analysis. Since the volumes and the concentration step for the calibration solutions and the filter recovery buffers were identical, the fungal α -amylase amount per mL in the calibration solution (ng/mL) is the same as the fungal α -amylase amount per filter (ng/filter).

Tryptic digestion

200 μ L of the concentrated protein solution was transferred into a 500 μ L tube. 2.5 μ L of 500 mM DTT was added and incubated for 30 min at 55 °C in a Thermomixer at 800 rpm to reduce disulfide bonds and then 2.5 μ L of 550 mM IAA in 100 mM ammonium bicarbonate was added and incubated for 30 min at room temperature in a Thermomixer at 800 rpm in the dark to alkylate cysteine residues.

10 μ L of 250 μ g/mL trypsin solution in 0.05 N HCl was added and digestion was performed for 16 h at 37 °C in a Thermomixer at 800 rpm. After digestion, the samples were centrifuged for 5 min at 20,000 g. 100 μ L of the supernatant was transferred into an injection vial and 5 μ L of formic acid was added.

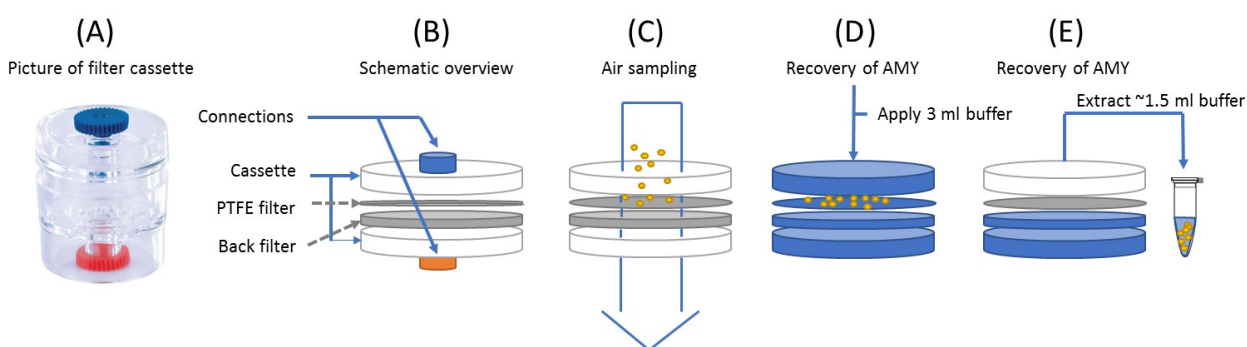


Figure 1. Overview of airborne dust sampling using PTFE filters (Millipore, FALP03700) and recovery of fungal α -amylase (AMY) from the filters. (A) Photograph of the filter cassette; (B) identification of the different parts of the filter cassette; (C) air sampling at different locations in the production facility; (D) 3 mL of buffer containing BSA was added and cassettes were mixed thoroughly; (E) 1.5 mL of buffer was removed from the cassette. The remaining buffer solution was absorbed by the back filter.

LC-MS/MS analysis

Peptide mixtures were analyzed with a Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer coupled to a Vanquish UHPLC system (Thermo Fisher Scientific). For peptide separation, a Waters ACQUITY CSH C18 column (10 cm length, 1 mm inner diameter, 130 Å pore size, and 1.7 μ m particle size) was used. The column was operated at 50 °C. Eluents were LC-MS-grade water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). Peptides were eluted with a linear gradient from 2 to 40% B for 21 min, 40 to 70% B for 1 min, and 70% B for 3 min at a flow rate of 300 μ L/min. Then the mobile phase was reset to the starting condition (2% B) within 0.1 min and equilibrated for 5 min before the next run. The sample injection volume was 25 μ L. The Q Exactive Plus was operated in selected ion monitoring (tSIM) mode using high-resolution accurate-mass (HR-AM) scanning at a resolution of 17,500. tSIM was set up using an inclusion list for the fungal α -amylase peptides: LIASANAIR m/z 464.7824 RT 3.8 min, DTGFVITYK m/z 465.7320 RT 4.1 min, FASYTNDIALAK m/z 657.3404 RT 6.5 min, GTDSGQIVTILSNK m/z 716.8857 RT 7.5 min, and SDC*PDSTLLGTFVENHDNPR*[1xCarbamidomethyl] m/z 758.6746 RT 8.4 min, all with a 1 min retention time window. All m/z ratios used are the doubly or triply charged species of the individual peptides. Results were filtered for peptide accurate mass (mass accuracy <5 ppm) and correct retention time (retention time accuracy within 0.2 min).

Tryptic peptides from fungal α -amylase that were used in the tSIM inclusion list were selected based on the highest concentration of fungal α -amylase standard measured. This standard was measured in a HCD Top 3 mode with dynamic selection of the three most intense peaks from each survey scan (m/z 400-1600) with a collision energy of 27 eV for fragmentation. The resolution for full scan (m/z 400-1600) and MS/MS scan was 17,500. Dynamic exclusion time was 10 s. The top 5 most intense peptide signals for fungal α -amylase were manually selected and identity was confirmed based on MS intact precursor mass and MS/MS fragmentation spectrum (mass accuracy 5 ppm) as well as a database search. Database searching was performed using Proteome Discoverer 2.1 (Thermo Fisher Scientific). MS/MS spectra were searched against an in-house database containing the amino acid sequences of BSA and α -amylase. Tandem MS data were searched using Sequest HT search engine with the following parameters: precursor mass tolerance of 5 ppm, fragment tolerance of 0.05 Da, trypsin specificity with a maximum of 2 missed cleavages, cysteine carbamidomethylation, methionine oxidation and deamidation of asparagine and glutamine as variable modifications. False discovery rate (FDR) was estimated by running the searches against a randomized decoy database. Results of the identification step were filtered to proteins with FDR below 1%. Based on accurate precursor mass, fragmentation spectrum and retention time this confirms undoubtedly the identity of the selected peptides.

RESULTS AND DISCUSSION

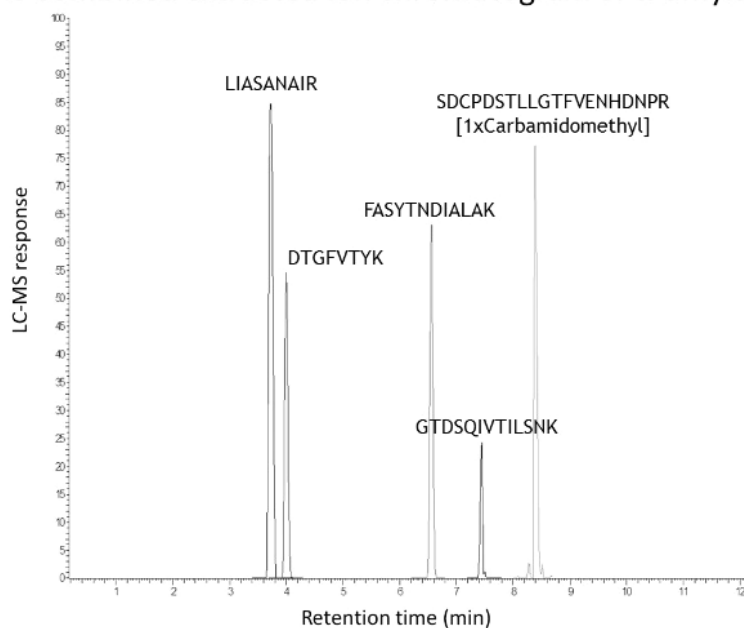
A HR-AM tSIM LC-MS method was used to detect and quantify fungal α -amylase. Peptides were generated after protein reduction, alkylation of cysteines, and digestion using the enzyme trypsin.

The reproducibility of the sample preparation and the LC-MS method was confirmed by analyzing the 3 most abundant tryptic peptides of BSA, which were added in a fixed concentration to

control the total analysis, and were detected with a relative standard deviation (RSD) of $\sim 10\%$ ($n = 7$). This confirms that the sample preparation was performed in a reproducible way. It was thereby assumed that the same RSD level applies for α -amylase from the filter samples.

The calibration curve (Figure 2) showed a linear relationship between the standard protein concentration and the average MS intensities of five peptides

LC-MS combined extracted ion chromatogram of α -amylase peptides



α -amylase calibration curve

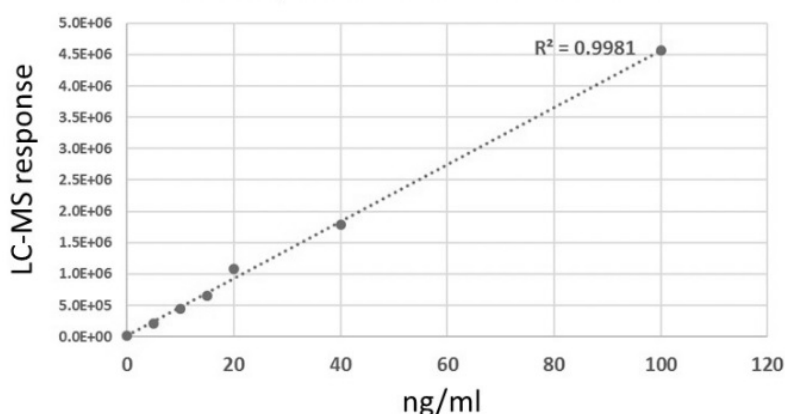


Figure 2. Combined extracted ion chromatogram for fungal α -amylase peptides in calibration standard of 20 ng/mL (top). Calibration curve of fungal α -amylase (bottom). This curve was based on the response of five selected peptides LIASANAIR, DTGFVITYK, FASYTNDIALAK, GTDSQIVTILSNK and SDCPDSTLLGTFVENHDNPR in HR-AM t-SIM.

Table 1. Amount of fungal α -amylase detected in dust filters. Amounts were calculated based on an external calibration curve prepared using Fungamyl Ultra[®] with a fungal α -amylase content of 650 mg/g.

Filter	Description	Fungal α -amylase (ng/filter)	Air sampled (m ³)	Fungal α -amylase (ng/m ³) ¹
1	Operator 1	5	0.43	12
2	Operator 2	55	0.42	130
3	Siever	<5	0.16	<30
4	Glove box	<5	0.16	<30
5	Automatic sampler (bottom mixer)	<5	0.23	<20
6	Packaging line (welds control)	<5	0.25	<20

¹The amount (ng) of fungal α -amylase/m³ was calculated from the amount (ng) of fungal α -amylase/filter divided by the volume (m³) of air sampled.

(for peptides selected, see Material and Methods). Fungal α -amylase levels down to 5 ng/filter could be quantified.

Fungal α -amylase amounts were calculated for all the filters. The same fungal α -amylase concentrations were measured in the solutions obtained from the top of the filter cassettes and from the back filters, indicating that fungal α -amylase was uniformly distributed throughout the interior of the filter cassette after dissolution in recovery buffer. As shown in Table 1, fungal α -amylase was detected on filters 1 and 2. These filters were carried by operators 1 and 2. These data show that the operators were exposed to fungal α -amylase in the enzyme product formulation process. Fungal α -amylase levels were >10 ng/m³ in the operator working area. No signal was measured for selected fungal α -amylase peptides on all the other filters.

CONCLUSION

A LC-MS/MS method was developed for quantification of α -amylase of *Aspergillus* origin by airborne dust sampling on filters at a workplace. A good linear relationship ($R^2 = 0.9981$) was obtained between standard protein concentration and the average MS intensities of five tryptic peptides selected. The limit of quantification (LOQ) of the method was 5 ng/filter. The assay can be used to monitor amylase exposure directly at the workplace and, therefore, may be

instrumental in optimizing work processes in production facilities. Knowledge of actual levels of allergen exposure allows timely protective measures to be taken to prevent further contact with the allergen and possible sensitization to the enzyme. As an additional advantage, the possibility of measuring actual levels of airborne allergens would contribute to a better awareness of the people at risk, i.e., the operators in a production plant.

CONFLICT OF INTEREST STATEMENT

None of the authors has a conflict of interest in any aspects of this work.

REFERENCES

1. Flindt, M. L. 1979, Lancet, 1, 1407-1408.
2. Sandiford, C. P., Tee, R. D. and Taylor, A. J. 1994, Clin. Exp. Allergy, 24, 549-557.
3. Losada, E., Hinojosa, M., Quirce, S., Sánchez-Cano, M. and Moneo, I. 1992, J. Allergy Clin. Immunol., 89, 118-125.
4. Vanhanen, M., Tuomi, T., Tiikkainen, U., Tupasela, O., Tuomainen, A., Luukkonen, R. and Nordman, H. 2001, Occup. Environ. Med., 58, 119-123.
5. Vanhanen, M., Tuomi, T., Nordman, H., Tupasela, O., Holmberg, P. C., Miettinen, M., Mutanen, P. and Leisola, M. 1997, Scand. J. Work Environ. Health, 23, 385-391.

6. Houba, R., van Run, P., Doekes, G., Heederik, D. and Spithoven, J. 1997, *J. Allergy Clin. Immunol.*, 99, 286-292.
7. Sander, I., Neuhaus-Schröder, C., Borowitzki, G., Baur, X. and Raulf-Heimsoth, M. 1997, *J. Immunol. Methods*, 210, 93-101.
8. Koets, M., Sander, I., Bogdanovic, J., Doekes, G. and van Amerongen, A. 2006, *J. Environ. Monit.*, 8, 942-946.
9. Bogdanovic, J., Koets, M., Sander, I., Wouters, I., Meijster, T., Heederik, D., van Amerongen, A. and Doekes, G. 2006, *J. Allergy Clin. Immunol.*, 118, 1157-1163.
10. Di Filippo, P., Pomata, D., Riccardi, C., Buiarelli, F., Gallo, V. and Quaranta, A. 2014, *Atmos. Environ.*, 98, 179-189.
11. Matos, J. T. V., Duarte, R. M. B. O. and Duarte, A. C. 2016, *TrAC Trends Anal. Chem.*, 75, 97-107.
12. Liu, F., Lai, S., Reinmuth-Selzle, K., Scheel, J. F., Fröhlich-Nowoisky, J., Després, V. R., Hoffmann, T., Pöschl, U. and Kampf, J. 2016, *Anal. Bioanal. Chem.*, 408, 6337-6348.