

George F. Schappi^a
Christian Monn^a
Brunello Wuthrich^b
Hans-Urs Wanner^a

^a Institute of Hygiene and Applied
Physiology, Swiss Federal Institute
of Technology and

^b Allergy Unit, Department of Dermatology,
University Hospital, Zurich, Switzerland

Direct Determination of Allergens in Ambient Aerosols: Methodological Aspects

Key Words

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Abstract

In Switzerland the birch tree (*Betula verrucosa*) major allergen Bet v 1 and the grass (*Phleum pratense*) pollen major allergen Phl p 5 are of particular relevance for inducing pollinosis. In this study, the protein and major allergen contents of aerosols of different aerodynamic diameters were determined. The aerosols were sampled by Andersen-Impactors and submitted to protein assays and allergen assays (ELISA) specific for Bet v 1 and Phl p 5. The total protein, Bet v 1 and Phl p 5 concentrations were correlated with the corresponding pollen counts. The presence of Bet v 1 in smaller aerosol fractions was demonstrated before and after birch pollen was counted, especially in the lower particle size ranges.

Introduction

The prevalence of allergic diseases has increased over recent years [1]. In Switzerland, 10–20% of the population suffer from allergic diseases, mainly hay fever. The most recent epidemiological studies have shown prevalence rates of 11.1% for allergic rhinitis (hay fever) alone [2]. Proteins in aerosols originate to a certain extent from pollen and pollen fragments. Water-soluble proteins are particularly relevant to allergic reactions [3, 4]. Determination of the total protein concentration in aqueous extracts of sampling filters may provide a means to estimate the allergenic potential. So-called major allergens amongst the allergy-relevant proteins are responsible for inducing most pollen allergies. In Switzerland the birch tree (*Betula verrucosa*) major allergen Bet v 1 and the grass (*Phleum pratense*) pollen major allergen Phl p 5 are of particular relevance [2]. Therefore, determination of the concentration of these two major allergens is of great interest.

Some studies have shown that allergens from pollen can be found in particle size fractions much smaller than un-

damaged pollen (smaller than 5 µm) [5, 6], and pollen fragments or released allergens may account for this observation. Several fragmentation or release mechanisms are postulated, for example rupture of the pollen by osmotic shock, or enhanced allergen release by interaction with airborne particulate matter [7, 8]. This may explain allergic reactions in parts of the lungs which cannot be reached by undamaged pollen because of their aerodynamic diameter (in general distinctly larger than 10 µm [9]). Particles in sizes below 10 µm aerodynamic diameter are small enough to penetrate the thoracic region (the tracheobronchial as well as the alveolar regions) of the human respiratory tract. The deposition of the inhaled particles increases in proportion to the square of their diameter. Thus, the possibility of penetration into progressively deeper compartments of the lung increases with decreasing aerodynamic diameter. In the tracheobronchial region a large proportion of the particles with an aerodynamic diameter of about 5–10 µm is deposited, in the alveolar region a large proportion of the particles with an aerodynamic of about 1–5 and <0.1 µm. The total deposition of particles between 0.1 and 1 µm is, however, quite low

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Correspondence to: Dr. Georg Schappi
Institute of Hygiene and Applied Physiology
Swiss Federal Institute of Technology
Clausiusstr. 25
CH–8092 Zurich (Switzerland)

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because of the low relative importance of the deposition mechanisms (gravitational deposition and impaction) for this particle size class [10].

However, the inhalable and thus biologically active fractions of the ambient aerosol have not been well characterized so far [11]. This investigation aims at answering the following questions. (1) What protein concentrations (originating from e.g. pollen and pollen fragments) can be found in the different particle size fractions? Is there a correlation with the corresponding pollen counts? (2) What specific birch and grass major allergen concentrations can be found in the different particle size fractions? Is there a correlation with the corresponding birch and grass pollen counts? The experimental design chosen made it possible to characterize the temporal progress of the particulate mass, total protein, Bet v 1 and Phl p 5 concentrations in different particle size fractions.

At the sampling site pollen was routinely counted and differentiated according to species (Swiss National Pollen Observation Program, NAPOL). It was therefore possible to compare the total protein concentrations with the corresponding sum of the pollen counts, as well as the total Bet v 1 and Phl p 5 concentrations with the corresponding sum of birch and grass pollen counts.

Materials and Methods

Sampling of Particles, Especially of Pollen and Pollen Fragments

Sampling was carried out by means of Andersen-Impactors (Andersen, Atlanta, Ga., USA). Andersen-Impactors are cascade impactors and collect particles of different size fractions at different stages. At the first stage an airstream (28.5 litres per minute) is led through the openings of a well-defined diameter. The airstream hits a sampling medium (glass fiber filter with a radius of 40.5 mm) positioned beneath. Particles of a well-defined aerodynamic diameter are deposited on the filter by impaction. The airstream carrying particles not deposited on the first filter is then led through the openings of the following stages. Successive reductions in the aperture diameter result in increased stream velocity and particles of smaller aerodynamic diameter are deposited on the filters at each stage. So different size fractions of particles are successively deposited at various stages. Particles with an aerodynamic diameter of above 9 µm are collected at the first stage, particle size fractions of 9–5.8, 5.8–4.7, 4.7–3.3, 3.3–2.1, 2.1–1.1, 1.1–0.65, 0.65–0.43 and <0.43 µm at the following stages. In this way the behavior of particles in the human respiratory tract is simulated: large particles are deposited in nose and mouth, smaller particles are able to penetrate into progressively deeper compartments of the lung.

Over 1 year (1994) sampling was continuously carried out at the Swiss Meteorological Institute in Zurich, Switzerland, at a height of about 10 m above ground. The 2-week sampling period chosen represents a compromise between a high resolution in time and the accumulation of an adequate quantity of material for analysis. Particle mass concentrations were determined gravimetrically. Glass fiber filters were dried for 24 h, conditioned for 24 h (at room temperature and

52% relative humidity) and weighed (Mettler AT 261 Deltarange) before and after exposure [12]. Parallel sampling showed that this procedure allowed a mass concentration determination with a precision of $\pm 10\%$. After gravimetric analysis the exposed filters were divided into two parts and analyzed as follows.

Quantification of Proteins in Different Particle Size Fractions

The filters from two successive stages were pooled in order to get higher concentrations. The resulting pools contained particle fractions of >9, 9–4.7, 4.7–2.1, 2.1–0.65, and <0.65 µm. For the extraction of water-soluble proteins the exposed filters from two successive stages were put into 7.5 ml of water (ultrahigh purity quality, pH 5.5) and were moved overnight on a shaker at 4°C. The extracts were then centrifuged at 5,500 rpm to eliminate suspended particles. The total protein concentration in different size fractions was determined by means of a standardized protein assay according to Bradford [13]. The system was calibrated with bovine γ -globulin. Under these conditions proteins and polypeptides with a molecular weight of above 3,000 daltons could be detected. Determining of the protein concentration of every sample was performed twice. This procedure accordingly allowed a precision of $\pm 9\%$.

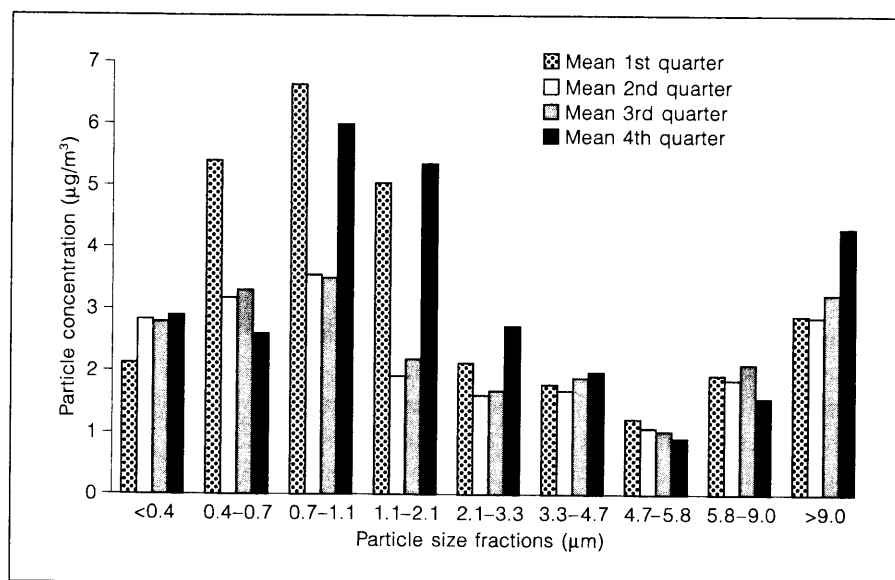
Determination of Immunologically Relevant Antigens

Bet v 1 and Phl p 5

The exposed filters were submitted to specific allergen assays (enzyme-linked immunosorbent assay, ELISA) against the birch tree major allergen Bet v 1 and the grass major allergen Phl p 5, respectively. Monoclonal antibody-based two-site binding assays are used to standardize allergen extracts in terms of quantification of single allergens [14]. We used this kind of assay, kindly provided by Allergopharma Joachim Ganzer, Reinbek, near Hamburg, Germany, to quantify Bet v 1 and Phl p 5, major allergens of birch pollen, and *P. pratense* pollen, respectively. The assays were adapted to quantify the allergens directly after extraction from particulate filters. The filters from two successive stages were put into 7.5 ml of a 0.125 M NH_4HCO_3 /0.1% BSA buffer and were moved overnight on a vortexer at 4°C. The resulting extracts were lyophilized under centrifugation for 3 h (Univapo Concentrator Centrifuge 150 AS Autospin, UniEquip Martinsried/München, Germany) and stored at -80°C until analysis. This procedure produced higher concentration ranges.

Quantification of Bet v 1. 60% of the lyophilisate from every sample was dissolved in 150 µl phosphate-buffered saline, containing 0.05% v/v Tween-20 (TPBS) and 0.01% thimerosal. 100 µl of purified monoclonal antibodies 3B4F11D6 against Bet v 1 at 10 µg/ml in a sodium carbonate buffer were added to each well of a microtiter plate and incubated overnight at 4°C (coating). All subsequent incubations were performed at room temperature. After blocking with TPBS/Thimerosal and two subsequent washes, 100 µl of Bet v 1 standard solution (affinity chromatography-purified birch pollen extract with defined allergen concentration; concentration range 1–1,000 ng/ml) and the samples were added to the wells. After 1 h of incubation the plates were washed 3 times and incubated with 100 µl of biotinylated monoclonal antibodies 2E10G6G7 1:1,000. Again after 1 h of incubation the plates were washed 3 times. 100 µl of streptavidin-peroxidase (Sigma, Deisenhofen, Germany) was added and incubated for 30 min. The Bet v 1 ELISA was developed with *o*-phenylenediamine as the substrate. The resulting absorption was quantified by means of a photospectrometer at 492 nm (Titertek Multiscan MCC/340). In order to verify the specificity of the monoclonal antibodies, the following allergen ex-

Fig. 1. Mean particle concentrations in the different size fractions (four quarters of 1994: winter, spring, summer, autumn) in Zurich.



tracts were submitted to Bet v 1 ELISA procedure: *Poa pratense* (Poa p 1), *P. pratense* (Phl p 5), *Lolium perenne* (Lol p 1), *Dactylis glomerata* (Dac g 1), *Alternaria alternata* (Alt a 1), *Corylus avellana* (Cor a 1), *Holcus lanatus*, *Festuca pratense*, *Plantago lanceolata* and *Artemisia vulgaris* pollen extract, kindly provided by Allergopharma Joachim Ganzer. The concentration of the allergen extracts was 500 µg protein/ml. The results indicated that these extracts do not interfere in the Bet v 1 ELISA. However, cross-reactive binding activity was found with *Alnus glutinosa* (Aln g 1) pollen extract.

Quantification of Phl p 5. 40% of the lyophilisate from every sample was dissolved in 100 µl TPBS. The subsequent steps of the Phl p 5 quantification method are described by Fahlbusch et al. [15, 16]. Repeated submission of the same sample to allergen quantification showed that this procedure allowed a precision of $\pm 10\%$. In cases where the Bet v 1 and Phl p 5 concentrations were in the lower range of the standard curve, the precision of the determination was reduced to $\pm 15\%$.

Pollen Counts

At the sampling site pollen of about 40 species were routinely collected with a Burkard Pollen Trap [17], counted and differentiated according to their species by means of a light microscope (Swiss National Pollen Observation Program, NAPOL).

Results

Concentrations of Particles in Different Size Fractions

The total particle concentration (annual mean for 1994; $n = 24$) was $25 \mu\text{g}/\text{m}^3$ in Zurich. Particle concentrations at different stages show characteristic patterns with maximum mass concentrations for particles with aerodynamic diameters of about $1 \mu\text{m}$ (accumulation range) and of $>9 \mu\text{m}$. In

the winter and autumn, the mass concentration in the important size range of between 0.7 and $2.1 \mu\text{m}$ was about twice as high as in the spring and summer. Figure 1 shows the mean particle concentrations in different size fractions for the four quarters of 1994 (winter, spring, summer, autumn).

Total Protein Concentrations in Different Size Fractions

The total protein concentration (annual mean for 1994; $n = 24$) was $540 \text{ ng}/\text{m}^3$ in Zurich. Protein was identified in all particle sizes. Sampling periods with high pollen concentrations were characterized by high protein concentrations in the aerosol fraction with the largest aerodynamic diameter. Protein concentrations at different stages show characteristic patterns with maxima for particle size fractions with aerodynamic diameters of about $<0.7 \mu\text{m}$ and of $>9 \mu\text{m}$. Figure 2 shows the mean protein concentrations in different pooled size fractions for the four quarters of 1994.

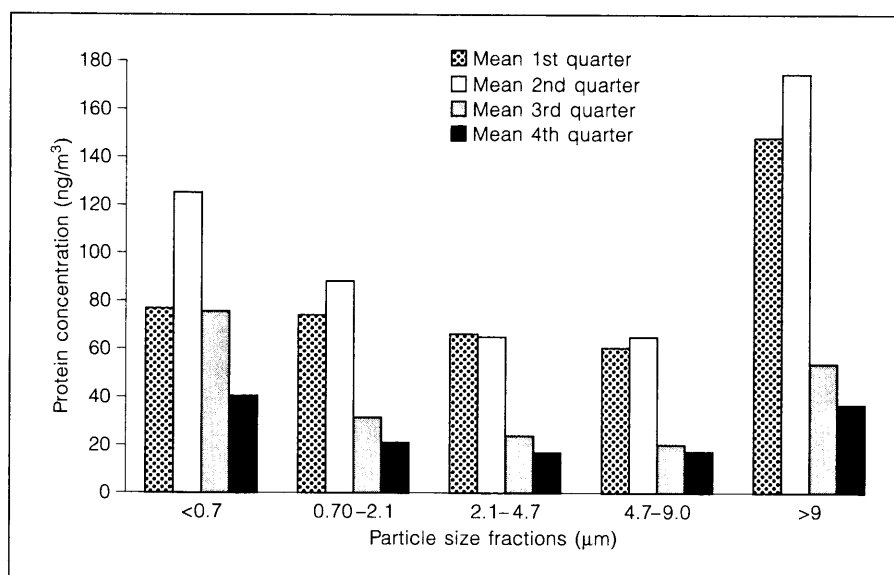
Comparison of Total Protein Concentrations with Pollen Counts

The 2-week means of total protein concentrations were correlated with the corresponding sum of the pollen counts. The correlation for 1994 is presented in figure 3. The regression corresponds to the equation $y = 393 + 1.5 \cdot x$ ($r^2 = 0.58$).

Major Allergen Bet v 1 and Phl p 5 Concentrations in Different Size Fractions

Bet v 1 and Phl p 5 were identified in all particle size ranges, including the smallest fractions. Sampling periods with high birch and grass pollen quantities were character-

Fig. 2. Mean protein concentrations in the different pooled size fractions (four quarters of 1994: winter, spring, summer, autumn) in Zurich.



ized by elevated Bet v 1 and Phl p 5 concentrations in the aerosol fraction with the largest aerodynamic diameter. Birch pollen was counted from the 21st March until the 18th May. Bet v 1 was detected, especially in the lower particle size ranges, before and after the birch pollen was counted. The existence of Phl p 5, however, was found only at the same time as grass pollen was counted. Figure 4 shows the Bet v 1 concentrations in different pooled size fractions in 1994, figure 5 shows the Phl p 5 concentrations.

Comparison of Total Bet v 1 and Phl p 5 Concentrations with Corresponding Pollen Counts

The 2-week means of total Bet v 1 and Phl p 5 concentrations were compared with the corresponding sum of birch and grass pollen counts. The regression analysis for Bet v 1 concentration and birch pollen counts gives the equation: $y = 0.01 + 0.0005 \cdot x$ [y: Bet v 1 concentration (ng/m³), x: amount of birch pollen (counts); $r^2 = 0.8$]. The corresponding equation for Phl p 5 concentration and grass pollen counts is $y = 0.0004 \cdot x$ [y: Phl p 5 concentration (ng/m³), x: amount of grass pollen (counts); $r^2 = 0.6$].

Comparison of Total Protein Concentrations with Bet v 1 and Phl p 5 Concentrations

During the corresponding pollination period Bet v 1 represented a fraction of 0.07‰ (standard deviation = 0.04‰) of the total protein amount; Phl p 5 represented a fraction of 0.05‰ (standard deviation = 0.04‰).

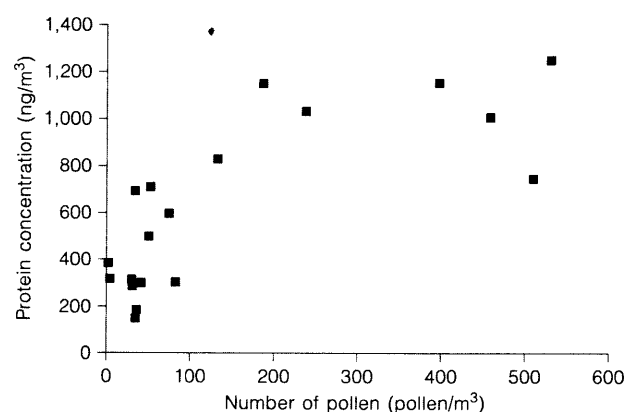


Fig. 3. Correlation between protein concentration and total number of pollen in Zurich (1994).

Discussion

One important finding was the correlation of the applied immunological methods with the corresponding pollen counts. The highest protein concentrations were detected in spring, corresponding to the largest total amount of pollen. As far as the correlation between the total protein concentrations and the total amount of pollen is concerned, the intercept of the regression equation indicates a constant background of proteins originating from other sources than

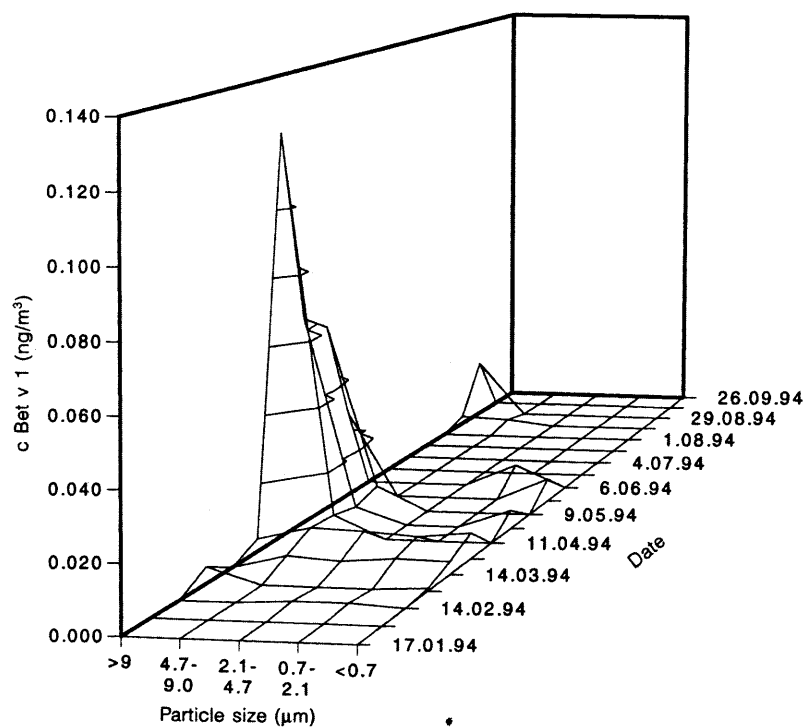


Fig. 4. Bet v 1 concentrations in different pooled size fractions in 1994.

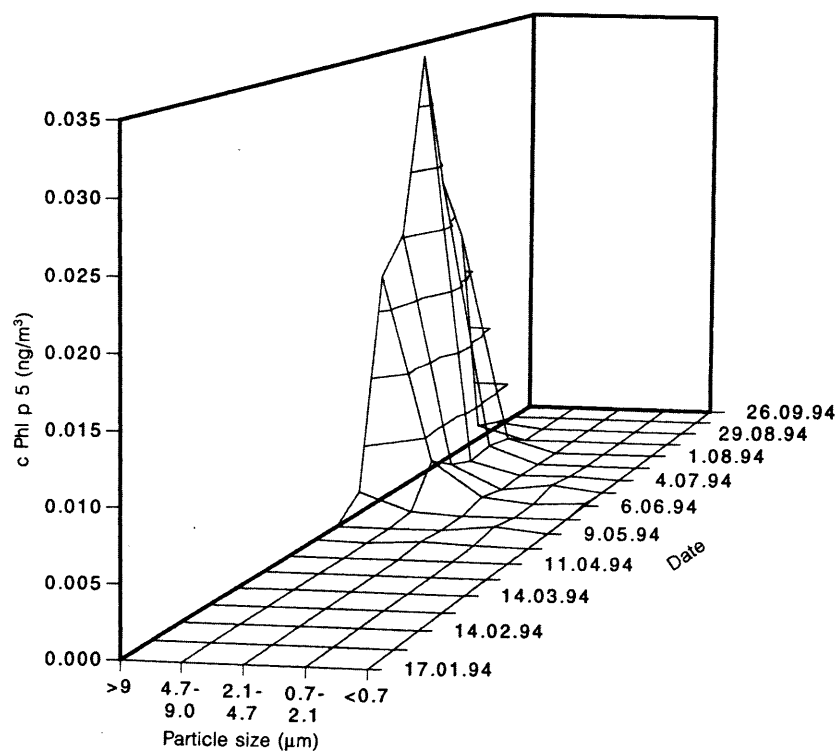


Fig. 5. Phl p 5 concentrations in different pooled size fractions in 1994.

pollen (for example spores, nonpollen plant material, microorganisms). The varying protein content of the pollen grains may be responsible for the deviations from a linear correlation [9].

The concentrations of the major allergens also principally corresponded to the detected birch and grass pollen amounts. However, the existence of Bet v 1 in smaller aerosol fractions was demonstrated before and after birch pollen was detected. This antigenic material may originate from bark, leaves and buds of birch trees [18] and may explain allergic symptoms before and after the birch tree pollination period. Furthermore cross-reactivities have to be taken into account. The antibody specificity tests indicated that the screened extracts (e.g. *C. avellana*, hazel pollen extract) do not interfere in the Bet v 1 ELISA. However, cross-reactive binding activity was found with *A. glutinosa* (alder tree) pollen extract. *A. glutinosa* is a related species from the same botanical family (*Betulaceae*). This cross-reactive binding activity may partly explain positive Bet v 1 ELISA results which did not correspond to the detected birch pollen. However, multiple regression analysis of total Bet v 1 with alder and birch tree pollen counts yielded a significance level of $p > 0.2$ for alder and $p < 0.01$ for birch tree pollen counts. Phl p 5 allergens were detected at the same time as grass pollen was found. Regression analysis of total Bet v 1 and Phl p 5 concentrations with the corresponding pollen counts yielded quite high correlations.

Another relevant aspect was that a certain portion of the protein and major allergen amount was detected in the particle size fraction with aerodynamic diameters of about $< 0.7 \mu\text{m}$, a fraction which is able to penetrate into the deep compartments of the lung (bronchioles, alveoli) [10]. Therefore, the allergens can produce immunological effects in very sensitive parts of the respiratory system. The existence of Bet v 1 and Phl p 5 in smaller aerosol fractions may suggest that there are fragments of birch and grass pollen or released pollen allergens [6, 7].

During the pollination period the protein and major allergen concentrations did not rise as rapidly as the corresponding pollen counts. This may suggest that the pollen proteins and major allergens were not extracted quantitatively, especially from intact pollen in the particle size fraction with an aerodynamic diameter of $> 9 \mu\text{m}$.

Because of an incompatibility of the reagents the extraction of proteins for the protein assay and the extraction of allergens for the ELISA could not be performed with the same buffer. This may cause different extraction efficiencies and has to be considered comparing the total protein concentrations with Bet v 1 and Phl p 5 concentrations.

Originally the applied Bet v 1 and Phl p 5 assays were used to standardize allergen extracts. In comparison to allergen extract concentrations, ambient allergen concentrations are usually very low. Thus an important methodological aspect was the necessity of getting higher sample concentrations for the ELISA procedure. This was achieved by lyophilization steps. Nevertheless there were still samples in the lower range of the standard curve which reduced the precision of the determination to $\pm 15\%$. The applied and partly newly developed methods seem to make the direct determination of proteins and major allergens in ambient aerosols possible. For the estimation of the allergy risk they may be a useful complementary approach to counting pollen. Future research aims at comparing aerosol samples originating from areas exposed to different extents of air pollution.

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