

Luminescence immunoassay of pollen allergens on air sampling polytetrafluoroethylene filters

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Abstract

A new method for quantification of airborne birch and grass pollen allergens collected on porous polytetrafluoroethylene filters has been developed. In this method, the allergens firmly adsorbed to the sampling filter of 25 mm in diameter are reacted with specific antibodies conjugated to alkaline phosphatase, generating a matrix-bound allergen–antibody–phosphatase complex. The filter is then floated on a chemiluminescent enzyme substrate solution. The light intensity of the product is linearly related to the amount of allergen over a large mass range, 0–1000 SQ (1 SQ is about 250 pg of protein). This direct on sampling filter in solution (DOSIS) technique demonstrated intra-assay precisions between 6–16% and 11–15% for the levels of 1–100 SQ units of grass allergen Phl p 5 and 4–400 SQ units of birch allergen Bet v 1, respectively. The limits of quantification for the corresponding allergens were estimated to 0.5 and 2 SQ units. Application of DOSIS to analysis of the grass pollen allergen concentrations of outdoor air for 12 days in July 1998 revealed a correlation coefficient of 0.69 between pollen grain and allergen concentrations for the dry weather period. After rainy days large amounts of grass allergens were present even in the absence of pollen grains. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

The quantification of pollen allergens in indoor air has been hampered by the lack of suitable techniques for quantitative entrapment of the allergenic particles as well as for their desorption from the sampling matrix. Recently it was demonstrated that birch pollen allergens in air are very efficiently adsorbed to porous polytetrafluoroethylene (PTFE) filters. However, it was not possible to extract these allergens with aqueous solutions which led to development of a new luminometric method for quantification of airborne birch pollen allergens collected on PTFE filters [1]. This direct on air sampling filter estimation (DOSAFE) technique, revealed that the air of school and office rooms may carry substantial amounts of birch and grass pollen allergens [2]. The principle of DOSAFE is that the target allergens, firmly bound to the PTFE sampling filter, are allowed to interact with specific antibodies conjugated with alkaline phosphatase. The sampling filter is subsequently soaked in a chemiluminescent alkaline phosphatase substrate and sandwiched between two sheets of transparency film. The light emitted as a result of the enzymatic activity of the allergen–antibody–phosphatase complex on the sampling filter membrane is recorded by a luminometer. Although very practicable, however, the DOSAFE technique has some inherent drawbacks. The logarithmic transformations of the experimental values necessary to obtain a linear relation between amount of allergen and luminometric response, *inter alia* disguise a low sensitivity of the method in the middle and high mass value ranges of the reported calibration curve; this is probably due to enzyme substrate exhaustion phenomena. Furthermore, the ratio of the amount of collected pollen allergen to sampling filter area available for the luminometric reading is low. Only about 2% of the total sampling filter area is scanned by the luminometer at each point of determination [1]. To overcome these limitations an improved and less laborious luminescence immunoassay for direct quantification of pollen-derived allergenic proteins adsorbed to air sampling PTFE filters was developed. This method yields a direct linear relationship between the amounts of purified major birch and grass pollen allergens used as standards and recorded luminescence from the whole sampling filter area over a large allergen mass range. Herein this direct on sampling filter in solution (DOSIS) technique for quantification of airborne birch and grass pollen allergens is described in detail.

2. Experimental

2.1. Materials

Water extracts of pollen from birch (*Betula pendula*) and timothy grass (*Phleum pratense* L.) for skin prick testing, Soluprick SQ[®], each containing 100 000 SQ units/ml (manufacturer's specification) were purchased from ALK A/S (Hørsholm, Denmark) and used as reference allergens for the standard curves. Polyclonal primary antibodies, rabbit IgG anti-*Betula* and rabbit IgG anti-*Phleum* were generous gifts from the same company. The birch and grass pollen extracts contained 23 µg Bet v 1 and 25 µg Phl p 5 protein/ml as major allergens, respectively, according to the manufacturer. All chemi-

icals were of analytical grades. Incubation media and reagents were prepared with ultrapure water of $18.2 \text{ M}\Omega \cdot \text{cm}$ resistivity, produced by a Milli-Q Plus system (Millipore, Bedford, MA, USA). The secondary antibodies, alkaline phosphatase-conjugated swine IgG anti-rabbit IgG were obtained from Dakopatts (Gentofte, Denmark). Polytetrafluoroethylene (PTFE, TeflonTM) filters, 25 mm in diameter with 0.2- or 1.2- μm nominal pore sizes and translucent polycarbonate 25-mm syringe filter holders (Cat.No. 16517E) were purchased from Sartorius (Göttingen, Germany). The filter holder was modified by removing the Luer lock inlet plastic part and attached filter support leaving an opening of 23 mm in diameter. Polystyrene Multidish six-well Nunclone Delta SI vials and polypropylene Nunc-Immuno $70 \times 11 \text{ mm}$ Minisorp tubes for preparation of allergen standards were obtained from Nunc (Roskilde, Denmark). Luminometric measurements were performed using a Luminoskan instrument and White Cliniplates from Labsystems OY (Helsinki, Finland). In some experiment white Fluoro Nunc Poly Sorp plates from Nunc were used. Round paper filters of 30 mm in diameter were punched from Munktell OOH paper filters (Munktell, Grycksbo, Sweden). A formulation of the chemiluminescent alkaline phosphatase substrate 4-methoxy-4-(3-phosphatephenyl)spiro[1,2-dioxetane-3,2'-adamantane] disodium salt, Lumi-Phos Plus, was from Lumigen (Southfield, MI, USA). The BCIP/NBT reagents for specific staining of alkaline phosphatase–allergen complexes on the filters were obtained from Bio-Rad (Hercules, CA, USA). Reciprotor vacuum pumps for air sampling of pollen allergens were purchased from LECTROSTATIC (Skara, Sweden). Air-volume metering instruments were of the bellow gas meter type and were calibrated against a Gallus 2000 G 1,6 from Euromekanik AB (Göteborg, Sweden).

2.2. Methods

2.2.1. General methods

Quantification of pollen grains in outdoor air was performed using a Burkard Seven Day Pollen trap from Burkard Manufacturing (Rickmansworth, Hertfordshire, UK). The Pollen counts were kindly made by the Palynological Laboratory at the Swedish Museum of Natural History in Stockholm Sweden and were determined 5 km from the place of the present study using a trap positioned about 10 m above the ground $59^{\circ}30' \text{N}$ and $18^{\circ}03' \text{E}$. The outdoor air grass pollen allergens were collected on the PTFE filters placed in fixed filter holders protected from rain on the roof of the institute ($59^{\circ}20' \text{N}$ and $18^{\circ}00' \text{E}$) about 10 m above the ground. The air flow through the sampling filter was $5.67 \pm 0.64 \text{ l/min}$ for about 7 h. After completed sampling each filter was collected and stored for about 6 months in a sealed polystyrene plastic box at -30°C prior to analysis. Meteorological parameters were recorded each day between 18:00 and 18:00 o'clock for 24-h periods, which started the day before the pollen allergen sampling day. This was performed by the Swedish Meteorological and Hydrological Institute at Bromma-Stockholm, Sweden ($59^{\circ}35' \text{N}$ and $17^{\circ}95' \text{E}$). The PTFE filters were handled with a sharp-pointed pair of forceps. The wells of the multidish vials were emptied by aspiration with a Pasteur pipette connected to a water pump. Incubation and washing media were gently pipetted onto the walls of the wells to keep the PTFE filter to float on the surface of the the relevant incubation or washing medium taking care that no air

bubbles were entrapped under the filters. If not otherwise stated all incubations and washings were performed at 22°C with the vials covered by their matching lids. Incubation of the PTFE filters with LumiPhos Plus was made in the dark by circular shaking with 300 rpm on an IKA-Schüttler MTS 2 apparatus (Janke & Kunkel, Staufen i. Br., Germany). All other incubations and washings were performed with moderate longitudinal shaking. Luminometric endpoint readings were made for 3 s with no lag time. If not otherwise stated the values for standards and samples represent single determinations of individual filters.

2.2.2. Preparation of pollen allergen standard curves

2.2.2.1. Step 1

To each of the six wells of the multidish polystyrene vial, 300 µl of 2-propanol were pipetted followed by addition of a 0.2- or a 1.2-µm PTFE filter which was placed on the surface of the alcohol. After equilibration for 1 min, 5 ml of 0.15 M sodium chloride containing 20 mM Tris–hydrochloric acid, pH 7.2 (TBS), was gently layered under each filter allowing them to float on the surface of the liquid during the addition.

2.2.2.2. Step 2

After 15 min with moderate shaking each wetted PTFE filter was layered on a paper filter, 30 mm in diameter and soaked in 250 µl of TBS, one for each of the six wells of a second multidish vial representing a humidified box. Any air bubble entrapped between the PTFE and the paper filter was eliminated. Excess of TBS in the wells was finally removed by aspiration. The vials used in step 1 containing the alcohol–TBS solutions was saved and stored at 5°C overnight for subsequent incubations.

2.2.2.3. Step 3

For standard curves ranging from 0 to 1000 SQ units, birch or grass pollen extracts with a defined single concentration of major allergen were pipetted as multiples of 10-µl droplets on the surfaces of six different PTFE filters layered on the paper filters in the humidified multidish vial leaving the sixth filter as a blank. Alternatively, in some experiments the standard PTFE filters were prepared from equal volumes of different dilutions of the allergens. The humidified vial containing the PTFE filters with adsorbed reference allergens was stored at 5°C overnight.

2.2.2.4. Step 4

The following day each standard PTFE filter was transferred to the surface of the corresponding original 2-propanol–TBS solution in the series of the six wells of the multidish vial from step 1, used for wetting the PTFE filters. After incubation for 15 min each standard filter was turned upside down and incubated floating on the solution for 60 min.

2.2.2.5. Step 5

The propanol–TBS solution in each well of the vial was removed and substituted with 5 ml of 0.5 M sodium chloride in 20 mM Tris–hydrochloric acid, pH 7.2, containing

0.2% (w/v) Tween 20 (0.2% TBST). The vial with the floating filters was moderately shaken for 90 min for blocking of residual protein-binding sites.

2.2.2.6. Step 6

The 0.2% TBST was removed and replaced by 0.5 M sodium chloride containing 0.05% (w/v) Tween 20 in 20 mM Tris–hydrochloric acid, pH 7.2 (0.05% TBST). The vial was incubated with shaking as in step 5 for 15 min.

2.2.2.7. Step 7

The floating standard filters were each incubated with shaking for 60 min in 5 ml of a 1:1000 dilution of the rabbit IgG anti-*Betula* or rabbit IgG anti-*Phleum* in 0.05% TBST followed by three successive washings with 5 ml of 0.05% TBST for 15 min

2.2.2.8. Step 8

After completion of step 7, the standard filters were incubated with shaking on the surface of a 1:1000 dilution of swine IgG anti-rabbit IgG conjugated with alkaline phosphatase in 0.05% TBST for 60 min followed by three successive 5-ml washings with 0.05% TBST and a final one with 5 ml of TBS for 15 min each. The multidish vial containing the six standard filters was stored overnight at 5°C.

2.2.2.9. Step 9

The following day the filters were transferred to a new, third multidish six-well vial containing 5 ml of 0.1 M sodium chloride and 5 mM magnesium dichloride in 100 mM Tris–hydrochloric acid, pH 9.6, in each well. After equilibration for 15 min with moderate shaking, the buffer was removed and 1.0 ml of LumiPhos Plus was layered under each filter. The multidish vial with the set of six filters were incubated in the dark under kinetic control with shaking (see Section 2.2.1) for 30 or 60 min. Aliquots of 100 or 200 μ l of the substrate solution of each well were rapidly transferred to corresponding wells of the microplate for luminometric reading, starting 60 s after removal of the last aliquot.

2.2.3. Treatment of sample filters

The sample filters containing the adsorbed birch or grass pollen allergens were wetted, with the filter surface containing the allergens facing the air, with 2-propanol and TBS in a multidish six-well vial as described in step 1 for the preparation of standard curves (Section 2.2.2.1). After 15 min incubation with moderate shaking the filters were turned upside down and incubated for 60 min. Finally the filters were treated by the same procedures as described for preparation of standard curves, starting with step 5 (Section 2.2.2.5) where the filters are layered on the surfaces of the 2-propanol–TBS solutions.

3. Results

The present method of analysis of PTFE filters loaded with 0–1000 SQ units of purified major allergens of birch or grass pollen yielded standard curves which both

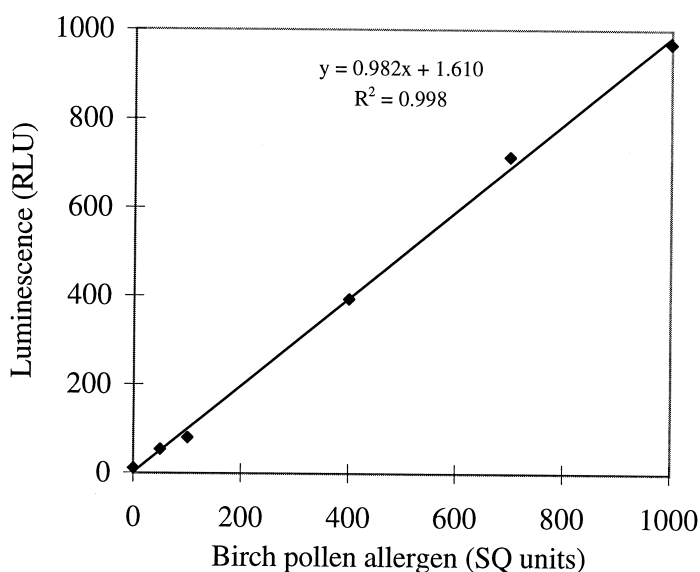


Fig. 1. Plot of amounts of the major birch pollen allergen Bet v 1 standard against luminescence intensities obtained with DOSIS for the 0–1000 SQ units range. The points of the calibration curve represent single determinations of the standard allergen pipetted on individual 1.2- μ m PTFE filters with diameters of 25 mm. The substrate sample size and incubation time was 100 μ l and 60 min, respectively.

showed linear relations between amounts of allergen and luminometric response, with correlation coefficients and intercepts close to 1 and 0, respectively, as depicted in Figs. 1 and 2. With 200- μ l samples of the luminescent substrate reaction mixture after incubation for 60 min, standard curves in the range of 0–10 and 0–1 SQ for the birch and grass allergens, respectively, were obtained (Figs. 3 and 4), however, at the cost of increased luminescence of the background. Loading the standard allergens on the PTFE filters as multiples of a fixed concentration or as different dilutions of a stock solution produced the same standard curves in the 0–1000 SQ units range. The 0.2- and 1.2- μ m PTFE filters did not differ significantly in allergen binding properties (not shown here). The intra-assay precisions of the method was estimated to 21, 16, 6 and 9% for the levels 0, 1, 10 and 100 SQ units, respectively, using grass pollen allergen and six different single filters at each level. Corresponding experiments with birch pollen allergen yielded 15, 11 and 14% intra-assay precisions for the levels 4, 40 and 400 SQ units, respectively. Analysis of single filters each carrying 100 SQ units of grass allergen on four and six different days gave inter-assay precisions of 17 and 32%, respectively. The limits of quantification were estimated to 0.5 and 2 SQ units per filter, which produced luminescence values that were larger than those of twice the background, for the grass and birch pollen allergens, respectively.

As an application of the present method for quantification of pollen allergens, outdoor air was sampled by means of PTFE filters for 12 days in July 1998 and analyzed for collected grass pollen allergens. As shown in Fig. 5 the concentrations of the allergens showed a big variation between days, between 26 and 176 SQ units per m^3 of air, as did

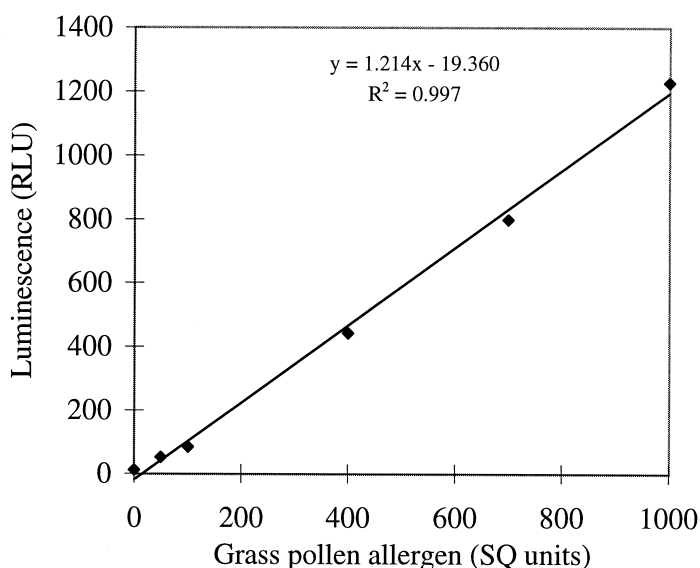


Fig. 2. Plot of amounts of the major grass pollen allergen Phl p 5 standard against luminescence intensities obtained with DOSIS for the 0–1000 SQ units range. The points of the calibration curve represent single determinations of the standard allergen pipetted on individual 1.2- μ m PTFE filters with diameters of 25 mm. The substrate sample size and incubation time was 100 μ l and 30 min, respectively.

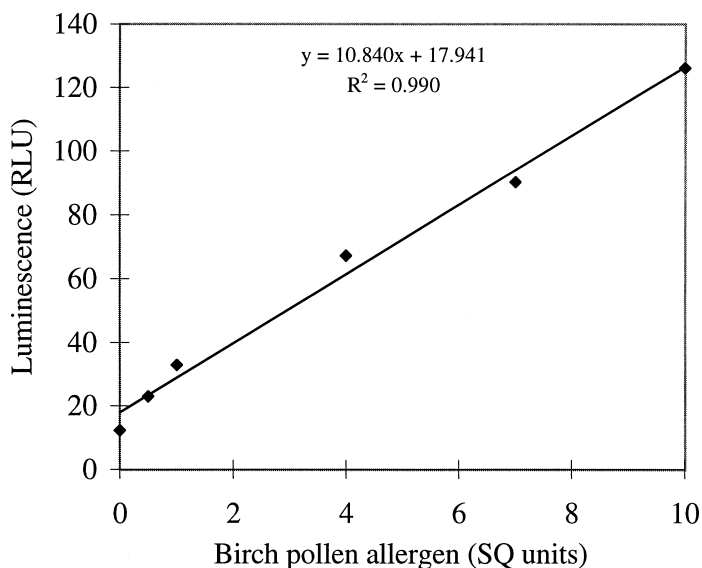


Fig. 3. Plot of amounts of the major birch pollen allergen Bet v 1 standard against luminescence intensities obtained with DOSIS for the 0–10 SQ units range. The points of the calibration curve represent means of duplicates of the standard allergen pipetted on individual 0.2- μ m PTFE filters with diameters of 25 mm. The substrate sample size and incubation time was 200 μ l and 60 min, respectively.

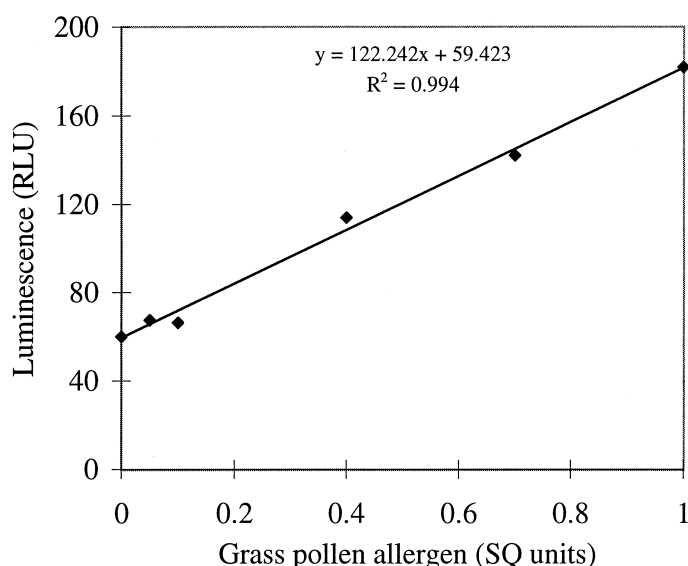


Fig. 4. Plot of amounts of the major birch pollen allergen Phl p 5 standard against luminescence intensities obtained with DOSIS for the 0–1 SQ units range. The points of the calibration curve represent means of duplicates of the standard allergen pipetted on individual 0.2- μ m PTFE filters with diameters of 25 mm. The luminometric readings were performed in White Fluoro Nunc Poly Sorp plates giving a higher background due to some cross-talk, than the White Cliniplates used in the other experiments. The substrate sample size and incubation time was 200 μ l and 60 min, respectively.

also the concentrations of pollen grains, between 0 and 22 grains per m^3 , determined by the Burkard trap for the same 8-h sampling periods. A plot of the concentration values for grass pollen allergens against their paired grass pollen grain concentrations, yielded a correlation coefficient of 0.40. However, if the two highest allergen values and the corresponding pollen counts for 6 and 9 July were excluded, the correlation coefficient increased to 0.69. Before these days, during the period from 4 to 6 July and between the seventh and eighth days, 45 and 15 mm of rain was falling, respectively, while the rest of the sampling period was dry. The precipitation seems to shift the peaks of the grass pollen allergen curve to later days as compared to those of the curve for the pollen grains (Fig. 5). Large amounts of grass pollen allergens were present in the air after the rainy days in concentrations of the same order of magnitude as previously reported [2]. Days with absent or with only a few pollen grains per m^3 of air showed substantial concentrations of grass pollen allergen as exemplified by the sixth, 13th and 15th sampling days in Fig. 5.

Staining of the sampling filters by the reagents BCIP/NBT specific for the alkaline phosphatase-conjugated antibody–allergen complexes, after luminometry, revealed the particulate nature of the air samples as illustrated in Fig. 6. The stain intensity of the filters by visual inspection agreed well with the corresponding luminescence intensity (Figs. 5 and 6).

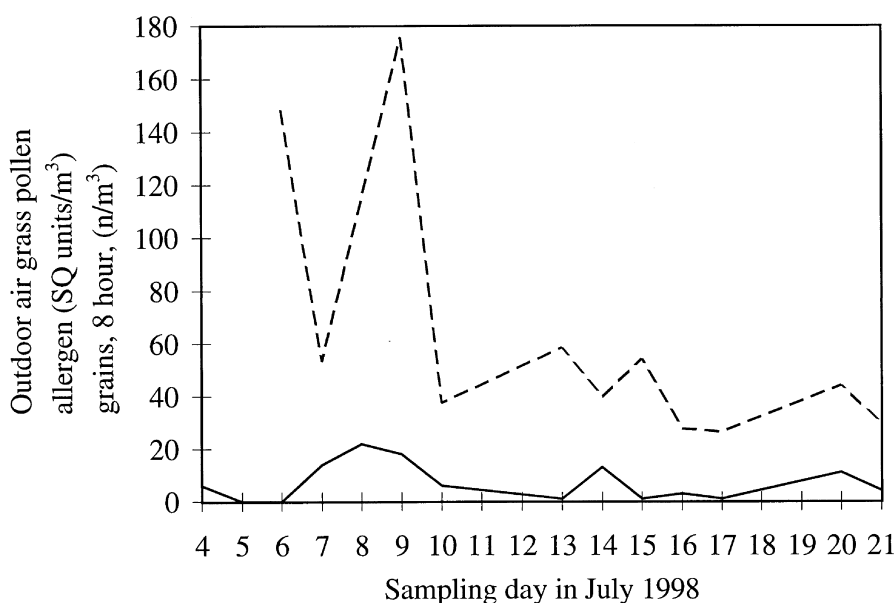


Fig. 5. Concentrations of outdoor air grass pollen grains obtained by the Burkard trap (solid line) and of outdoor air grass pollen allergens collected and determined by the DOSIS technique (dashed line) for 6–21 July 1998. The grass pollen grains were sampled and counted for the 8-h period covering the sampling time of allergens. The sampling conditions using 1.2- μm PTFE filters, 25 mm in diameter are as described in Section 2.2. The substrate sample size and incubation time was 100 μl and 30 min, respectively. Note that the pollen allergen concentration peak occurs delayed after the maximum of the pollen grain concentration on 8 July which was preceded by 15 mm of rain on the day before and by 45 mm before 6 July.

4. Discussion

The purpose of the present study was to improve the previously reported DOSAFE technique [1] for quantification of pollen allergens to yield a method that utilizes the whole sampling filter area and that gives the same sensitivity over the whole measuring range of the standards. This was accomplished by allowing the sampling filter containing the insolubilized allergen–phosphatase complex to float on an arbitrary volume of the substrate solution, instead of being sandwiched in a thin film of it as in DOSAFE, while generating the luminous product. Using the new method a large variation of both amounts and volumes is possible at the application of allergen standards as the whole filter area is available. According to the manufacturer the PTFE filter has the capacity to bind about 8 μg of protein per cm^2 which is far above the value for 1000 SQ units of grass or birch pollen allergen, corresponding to 0.25 μg of allergen protein distributed over the 25-mm filter, as used in the present study. Consequently smaller than previously used sampling filters of 47 and 50 mm in diameter, could be used in a less laborious analytical procedure entailing less consumption of reagents and antisera. The modification of the sampling filter holder described in the present report gives a very even distribution of the sampled pollen allergens on the filters (Fig. 6) as compared to

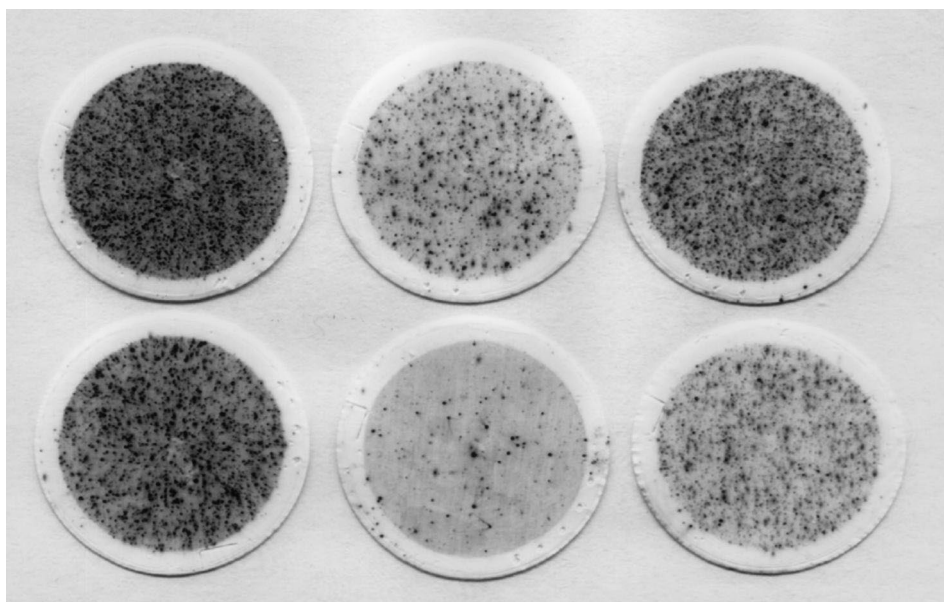


Fig. 6. Photograph of the first six 1.2- μ m sample filters with adsorbed grass pollen allergens producing the luminescence values of 6 to 11 July 1998, as shown in Fig. 5. The filters were submitted to specific BCIP/NBT-staining for allergen-immunoglobulin-alkaline phosphatase activity after the luminometric determination. The filters of the 6–8 and 9, 10 and the 13 July are arrayed from the left of the upper and lower rows, respectively. The white borders of the filters represent the areas covered by the rim of the filter holder inlet. Note the even distribution of stained dots originating from small and large particles over the whole filter area.

previously used holders [1]. As the present method demonstrates a linear relation between allergen concentrations and relative luminescence, explicit precision values can be obtained. The intra- and inter-assay precision values between 6 and 16% and 17 and 32%, respectively, obtained by single filter determinations are acceptable for the purpose to quantify pollen allergens in mapping and elimination studies in various indoor environments. These figures were obtained using six different individual filters for each calibration curve, in contrast to DOSAFE where 16 different dilutions of the allergen standard are pipetted onto the same filter. Thus, the new method also takes care of the variation related to the adsorbing properties of different sampling filters. If needed a higher precision can be obtained by running doublets or triplets of samples or standards. The limits of quantification of the birch and grass allergens are in agreement with those previously obtained [1]. DOSIS has an advantage of being based on the initial reaction velocity of the cleavage of a luminescent alkaline phosphatase substrate generating a luminous product. Furthermore the enzymatic reaction is stopped when the sample filter is removed from the substrate solution. The light emission was then fairly constant, decaying linearly with less than 2% per min at 22°C (not shown here).

It has been demonstrated that grass pollen grains might rupture in contact with rainwater with subsequent discharge of their particulate and allergenic content [3]. During rain the pollen grains are often washed down to the ground and when moist, rupture to release very small allergenic particles [3]. These might be airborne after periods of dry weather and dwell longer in the air than the pollen grains. Using the new method, DOSIS, a correlation coefficient of 0.69 between the concentrations in the air of grass pollen grains and their allergens was obtained for the dry period of the sampling days in July 1998. This indicates an expected connection between these parameters. However, as shown in Fig. 5, the grass pollen allergen concentrations increased on the days following rain in parallel with a decrease of the pollen grain concentrations. When including the rainy days in the correlation analysis the correlation coefficient decreased to 0.40. These findings and the grass pollen allergen concentrations in outdoor air in relation to those of pollen grains for the sampling period are in accordance with previously reported observations obtained by DOSAFE [1,2]. Using the DOSIS and DOSAFE techniques for pollen allergen quantification it can thus be demonstrated that the air may contain large amounts of grass pollen allergen particles, expressed as SQ, units even in the absence of pollen grains. Our studies of pollen allergens will continue.

5. A simplified description of the method and its (future) applications

A direct on sampling filter in solution (DOSIS) luminescence immunoassay for the quantification of airborne pollen allergens has been developed. This new technique involving also a newly designed filter holder is based on the previously reported DOSAFE principle, but the determinant quantification reaction is performed in solution instead of on the filter membrane. This has several advantages. Thus, DOSIS gives a direct linear relation between amounts or concentrations of allergen against luminescence intensities over a very large standard allergen range. The allergen mass to filter area ratio is high and the whole filter area is available for application of allergen standard in multiples of a fixed volume or in different concentrations. The sampling technique gives a very even distribution of large and small particles on the filter which reduces the risk for local excess of the allergen binding capacity of the PTFE filter. The DOSIS technique in addition requires less reagents, antisera and consumables and is less laborious as compared to the parent method. DOSIS will have applications to mapping and elimination studies of the air quality of various outdoor and indoor environments. This new analytical principle for quantification of airborne protein particles should also be applicable to other allergens from other sources.

Acknowledgements

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