

Optimization of the measurement of outdoor airborne allergens using a protein microarrays platform

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Abstract Increased knowledge on allergenic molecules in the environmental air helps in the information on environmental air quality and in the prevention and treatment of allergies. The aim of this study is to develop and validate a new methodology for the simultaneous detection and quantification of several airborne allergens using protein microarray technology, which has been created for the clinical detection of allergens. The immunological method was performed with Immuno Solid-phase Allergen Chip (ISAC) inhibition assay. Reagents for the validation studies include the following: (1) three sera from patients allergic to grass pollen each with different IgE levels as the detection reagents, (2) recombinant Phl

p 1 major allergen as the inhibitor for the inhibition assays, (3) “natural” Phl p 1 released by *Phleum pratense* (timothy grass) pollen grains as the “biologically” relevant aeroallergen and (4) samples of airborne pollens collected by a Multi-vial Cyclone Sampler for comparison of levels of pollen detection versus the protein allergen detection by the microarray assay. The results obtained showed that ISAC inhibition is a sensitive technique able to detect 2.1 pg/mL of Phl p 1 and the allergens released from 1 grain of natural pollen. Also, the airborne allergen samples analyzed showed a good correlation with the concentration of grass pollen in the air. The use of ISAC inhibition will greatly improve future airborne simultaneous allergen quantification, becoming a valuable option in air quality control.

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1 Introduction

Allergic diseases are a global health problem (Baiardini et al. 2010) and their incidence in respiratory diseases and asthma appears to be increasing worldwide (D’Amato et al. 2010). According to the European Community Respiratory Health Survey (ECRHS), the prevalence of specific IgE sensitization

to allergens in Europe, USA and Australia is about 35 % (Sunyer et al. 2004; Bousquet and Khaltaev 2007). Despite the relationship between airborne particles and allergic symptoms being established and documented (D'Amato et al. 2007), the amount and type of aerobiological components, geographical area, pollution, anthropic variations or eating habits determine the appearance of symptoms (D'Amato et al. 2007; Asero et al. 2009). A significant number of investigations over the last few years have been focusing on exploring and developing allergy prevention strategies that require integrated and multidisciplinary approaches (Samoliński et al. 2012; Gilissen et al. 2006).

Aerobiology is an important tool in quantifying the airborne emissions of pollen grains and spores by applying palynological techniques (Mandrioli et al. 1998) and helps in the prevention of respiratory allergy symptoms. It has been demonstrated that, when the airborne pollen grains or spores enter the respiratory tract, they release proteins that trigger the allergenic symptoms in the atopic population (D'Amato et al. 1998; Taylor et al. 2002). Then, knowing with accuracy the allergenic content in the air at any moment, allergy sufferers can adapt their treatment and avoid indiscriminate taking of medication. However, the aerobiological information does not completely explain all the pollinosis cases, as it does not detect the complete allergenic load in the atmosphere. Since 1981, several authors have shown interest in evaluating this allergenic load as a complement to the aerobiological studies, in order to better establish the periods of time with risk for the population (Argawal et al. 1981; D'Amato et al. 1996; Spieksma and Nikkels 1999; Cabrera et al. 2002; Moreno-Grau et al. 2006; De Linares et al. 2007, 2010a; Rodríguez-Rajo et al. 2011; Buters et al. 2012). In all these studies, the allergenic measurements were performed using immunological techniques, such as enzyme-linked immunosorbent assay (ELISA) or fluoroenzyme immunoassay (FEIA) tests.

Protein microarray technology has advanced remarkably in recent years, leading to the development of allergen chips for the detection and quantification of proteins in serum or other biological fluids (Jahn-Schmid et al. 2003; Harwanegg and Hiller 2005; Ebo et al. 2009; Rossi et al. 2007). The principle of these microarrays is a multi-analysis test that allows the simultaneous investigation of more than one hundred allergens in a single analytical step (Harwanegg and

Hiller 2005). Technically, the microarray results are comparable with traditional IgE assays such as ELISA or FEIA tests (Harwanegg and Hiller 2005; Ebo et al. 2009; Rossi et al. 2007; Wöhrle et al. 2006). However, protein microarrays offer several special features: the multiplexing methodology, amount of serum required, the solid-phase platform using very small quantities of individualized allergens and the high sensitivity for detecting small amounts of specific antibodies (Harwanegg and Hiller 2005).

This methodology has also been used for the detection of airborne allergen levels. Earle et al. (2007) evaluated indoor allergen exposure, and Heisler et al. (2009), De Linares et al. (2010b) and Belmonte et al. (2011) reported preliminary results of outdoor air quality. All concluded that protein microarrays methodology could be an accurate tool for verifying the indoor and outdoor air quality.

Like the studies of Hattori et al. (2011) that introduced the new concept of “microenvironment array chips”, the aim of our study is to validate the inhibition immunoassay using a protein microarrays platform (ISAC: Immuno Solid-phase Allergen Chip, Thermofisher Scientific Inc.) as a solid phase to quantify individual airborne allergens. Despite microarray platforms being produced include more than 100 different allergenic proteins, the validation of this technology cannot be globally evaluated taking into account all allergens together; thus, it was decided to evaluate the method using as model the protein Phl p 1. To achieve this, the following must be clarified: (a) the level of IgE in the serum of patients used in this study, (b) the detection limit (lowest concentration) of allergen that ISAC inhibition is able to detect, (c) the length of the hydration time needed for the pollen to release the allergen and (d) the relationship between the airborne allergen load measured and the aerobiological pollen counts.

2 Methods

2.1 Immunological technique for the quantification of allergens

2.1.1 Serum samples

The serum samples were obtained from the serum collection of the Parasitology and Immunoallergy

Table 1 Characteristics of the sera used in the inhibition assays

Allergenic source	Individual allergen	Specific IgE ISAC units in serum 1 (low)		Specific IgE ISAC units in serum 2 (medium)		Specific IgE ISAC units in serum 3 (high)	
		Undiluted	Working dilution	Undiluted	Working dilution	Undiluted	Working dilution
Timoty grass	rPhl p 1	13.0	2.0	20.0	5.0	117.0	15.0
	rPhl p 2	1.4	0.0	8.6	3.8	31.0	2.8
	nPhl p 4	6.2	0.0	0.0	0.0	18	4.8
	rPhl p 5	38.0	7.8	12.0	3.6	14	3.7
	rPhl p 6	0.0	0.0	7.1	1.4	0.0	0.0
Profilin	rPhl p 12	9.2	2.1	0.0	0.0	0.0	0.0

Data expressed in ISU (ISAC standardized units/L)

Laboratory, “Centro de Investigación y Estudios Avanzados, Lucio Lascaray”, of the Department of Immunology, Microbiology and Parasitology of the University of Basque Country, Vitoria (Spain). They were used according to a protocol approved by the Ethics Committee of the University of Basque Country, Vitoria (Spain). All patients gave their written informed consent prior to the study.

Human sera from patients sensitized to grass were used. The inclusion criteria were to exhibit a positive skin test and specific IgE >0.35 KU/L to timothy grass (*Phleum pratense* L.) and to show respiratory symptoms during the grasses pollen season. Three different sera were selected (Table 1), exhibiting different ISAC standard units (ISU) that were classified as low (2 ISU/L), medium (5 ISU/L) and high (15 ISU/L). These sera were used as reagents to perform the ISAC inhibition assays.

2.1.2 Inhibitors

Three different inhibitor samples were used to perform the inhibition studies:

1. Recombinant Grass Group 1 allergen (rPhl p 1) Bial-Aristegui S.L. (Bilbao, Spain) obtained in vitro from DNA sequence isolated from Group 1 Grass pollen and expressed in *E. coli* (Arilla et al. 2001). The inhibitor rPhl p 1 was used as a calibrator to measure the inhibition in the rest of the samples. In order to ensure the reproducibility, the calibration curves were performed in quintuplicate for each of the three selected sera at different times.

2. *Phleum pratense* pollen (Iberpolen S.L., Spain) was used to prepare different pollen grain concentrations (1, 5, 50, 500 and 5,000 pollen grains/mL) and each of them was submitted (in quintuplicate) to different hydration times (2, 4, 6, 12, 24 and 48 h). *P. pratense* pollen concentrations were used to determine, first, the optimal time of hydration for the pollen to release the allergens (inhibitors) and, second, the lowest pollen concentration that ISAC inhibition was able to detect.
3. Environmental airborne allergen samples obtained with a Multi-vial Cyclone Sampler were used as inhibitors to quantify the concentration of allergen in the air.

All inhibitor samples were reconstituted in 1 mL of PBS buffer (0.3 M NaCl, 10 mM phosphate, pH 7.4).

2.2 Inhibition assay using the allergen-chip platform

In the inhibition immunoassay, the allergen content in the unknown sample competes with solid-phase allergen to bind with specific IgE antibodies. The amount specific IgE bound to the solid-phase antigen is then measured. A standard curve, using different allergen concentrations in the sample (containing only PBS buffer, pH 7.4) as a reference, was plotted to calculate the amount of airborne allergens able to inhibit the IgE-allergen reaction in the solid phase.

The inhibition was performed by adding 10 μ L of each of the three sera to 10 μ L of each of the three inhibitors and incubating overnight at 4 °C. Control of proteolytic activity by SDS–PAGE electrophoresis did

not demonstrate differences between protein profiles of allergenic extracts before and after overnight incubation at 4 °C.

The presence of IgE was analyzed using the ISAC platform. ImmunoCAP ISAC (ThermoFisher Scientific) has developed a miniaturized immunoassay in a microarray format for the diagnosis of IgE-mediated type I allergic disease (ISAC test). A panel of selected recombinant and purified allergens is immobilized onto a glass surface (75 × 25 mm), compatible with the standard laboratory instrumentation. Each glass chip contains four arrays which include up to one hundred allergens localized in 400 individual spots, allowing in triplicate measurements to assure maximum assay reliability. Detailed ISAC assay protocol can be downloaded from the manufactures web page (www.vbc-genomics.com). According to manufacturer's guidelines, the microarray slides were washed for 60 min in a PBS-T buffer (150 mM sodium chloride, 10 mM Tris base and 0.5 % Tween20; pH 8.0) to rehydrate the solid phase and to remove non-covalently bound material from the microarray surface. Then, 20 µL of the competitive inhibitors were dispensed directly into each individual reaction well of the microplate. The slides were incubated for 120 min in a humid chamber. The excess inhibitor (coupled to part of the specific IgE) was removed by a washing step in a PBS-T buffer and distilled water. The competitive inhibition was monitored by adding 20 µL of a fluorescence-labelled anti-human IgE detection antibody. After 60-min incubation and a second washing step, the slides were scanned in a ScanArray GX PLUS Microarray Scanner (PerkinElmer Inc., USA). Visual display results were represented by fluorescence images that were analyzed using ScanArray Express Software (PerkinElmer Life Sciences Inc., USA). The fluorescence intensity values on the individual spots were quantified. Data were expressed as ISAC standardized units/L (ISU) that correspond to specific IgE antibody levels within a measuring range of 0.3–100 ISU-E (ImmunoCAP ISAC[®], ThermoFisher Scientific Inc.).

The percentage of inhibition (p) was calculated using the formula:

$$\% \text{ inhibition } (p) = [(IgE_0 - IgE_1)/IgE_0] \times 100,$$

where IgE₀ is the specific IgE value of the serum without inhibitor (100 % binding) and IgE₁ is the specific IgE value of the serum mixed with the

corresponding concentration of allergen inhibitor. Ag 50 is defined as the concentration of inhibitor able to inhibit the 50 % of the IgE antibody reactions with the antigen in solid phase.

2.3 Aerobiological methodologies

2.3.1 Allergen sampling collection

The airborne allergen samples were obtained using a Multi-vial Cyclone Sampler (Lippmann and Chan 1979) (Burkard Manufacturing Company Limited; England) at 23 m.a.s.l installed in the roof of Building C at the Universitat Autònoma de Barcelona, Spain. This sampler sucked 16.6 L/min of air that was injected into a 1.5-mL Eppendorf vial and allows the allergens to remain attached to the walls. This instrument offers an efficiency of 100 % for particle sizes up to 1.06 µm and 93.28 % for particle sizes 0.82–0.75 µm. The collector provides daily samples and ensures comparability of the allergen and airborne pollen data (Emberlin 1995). The samples were preserved at –80 °C until the analysis time and analyzed as explained in the previous section. The results of allergen concentration were expressed in picograms of allergen per cubic metre of air (pg/m³).

2.3.2 Pollen sampling collection

For the aerobiological pollen quantification, a Hirst (1952) volumetric collector (Lanzoni VPPS 2000, Italy) was used, located adjacent to the Multi-vial Cyclone Sampler. This collector aspires air at a known rate (10 L/min) and retains the pollen and spores adhered on a surface that was then cut into the corresponding daily samples. The samples were analyzed in accordance with the Spanish Aerobiological Network (Red Española de Aerobiología, REA) methodology (Galán et al. 2007) and the pollen data were expressed in pollen grains per cubic metre of air (Pollen/m³).

2.3.3 Comparison of airborne pollen and allergen measurements

Aerobiological pollen analyses were run without interruption. The airborne Poaceae pollen grains obtained were identified and quantified using an optical microscope in order to select 10 allergen samples corresponding to days with high, medium and low Poaceae

pollen concentrations. Additionally, 1 day without Poaceae pollen in the air was selected to be used as control. The comparison between aerobiological and allergen samples was then based on 11 cases.

2.4 Statistical analysis

The statistical analysis, based on regression equations with the percentage of inhibition (p) as the dependent variable and the concentrations of the rPhl p 1 and of pollen as independent variables, was performed with Stata Corp. 11 (2009). When the independent variable was the concentration of rPhl p 1 (log scale), a sigmoid curve based on a logit regression equation was used,

$$\ln(p/(100 - p)) = a + b \ln(C),$$

where C is the allergen concentration and p is the inhibition percentage. Dummy variables associated with the three serum samples and product terms of $\ln(C)$ and the dummies were also included in the logit regression equation. A single equation, estimated using ordinary least squares, could then be used for all the samples. The samples were compared testing the coefficients of the dummies and the product terms. The equality of the intercept (a) and slope (b) parameters across samples was separately tested with F statistics, to check whether the samples were interchangeable.

The sensitivity of the proposed technique was checked studying the linear portion of the detection curve both, when the independent variable was rPhl p 1 allergen and allergens released from different pollen concentrations. The reproducibility was measured by the coefficient of variation (CV) obtained by the five replicates (interassay) of all assays.

The detection limit is defined as the lowest amount or concentration of analyte in a sample which can be reliably detected (Long and Winefordner 1985). To calculate this, it is required the construction of analytical calibration curves expressed as:

$$\chi = mc + i$$

where m is the slope or analytical sensitivity and i is the intercept.

According to International Union of Pure and Applied Chemistry (IUPAC), the detection limit can be calculated by the blank values (10 replicates in our case). The detection signal limit (expressed as mean of blank values + 3 × standard deviation of blank values) is extrapolated to the linear calibration curve.

Moreover, we also calculated the limit of quantification defined as the lowest amount or concentration of analyte in a sample that can be reliably quantified with an acceptable level of precision and accuracy (Long and Winefordner 1985). The quantification signal limit (expressed as mean of blank values + 10 × standard deviation of blank values) is extrapolated to the linear calibration curve.

3 Results

3.1 Detection limits and variability of the inhibition assay used as reference; influence of the specific IgE concentration of the serum on the sensitivity of the inhibition immunoassay

Figure 1A shows the inhibition curves obtained from the dilution of each of the three sera incubated with different concentrations of the inhibitor rPhl p 1 in a solid-phase ISAC platform. The aim was to analyze the homology between the sera used as reference. In all cases, it showed that microarrays are highly sensitive; linear detection was calculated between 2 and 1,000 pg/mL. The detection limit was 2.3 pg/mL for the serum with 2 ISU, 2.1 pg/mL for the serum with 5 ISU and 1.9 pg/mL for the serum with 15 ISU (regression coefficients above 0.96 in the three models). The quantification limit obtained ranged between 2.4 pg/mL (for 2 ISU) and 1.9 pg/mL (for 15 ISU).

The statistical analysis, based on a logit regression (Fig. 1A), showed non-significant differences in both the slope ($p = 0.976$) and the constant ($p = 0.907$) parameters, thus supporting the inter-changeability of the sera for making the measurement of allergens in the airborne samples (regression coefficients above 0.90 in all cases). The CV was 10.7 % for the serum with 2 ISU, 17.7 % for the serum with 5 ISU and 17.3 % for the serum with 15 ISU.

To evaluate the influence of the specific IgE serum title, Ag50 was calculated using sera with different specific IgE concentrations. Inhibition immunoassays carried out on serum containing 2 ISU/L of specific IgE were able to inhibit 50 % of the solid-phase antigen-IgE antibody reaction using 6.9 pg/mL of inhibitor (rPhl p 1). For the serum containing 5 ISU/L, Ag50 was 6.1 pg/mL, and for 15 ISU/L, it was 5.4 pg/mL (Fig. 1A).

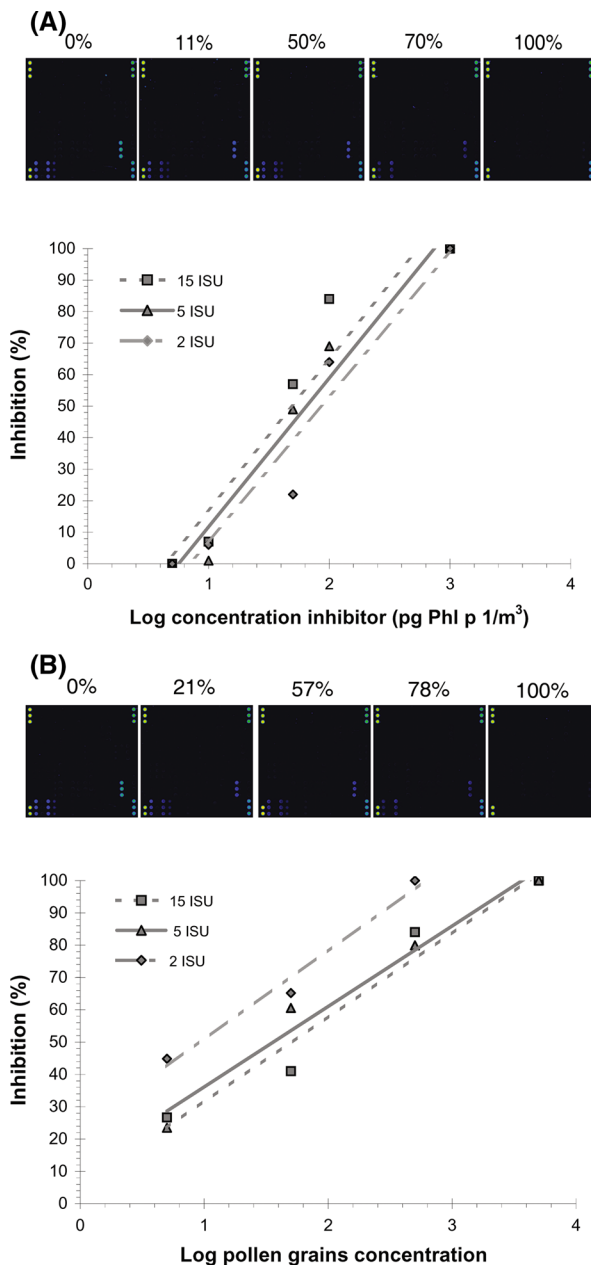


Fig. 1 **A** Reference assay using rPhl p 1 as inhibitor; **B** Pollen sample assay using *P. pratense* pollen as inhibitor. Examples of fluorescence micrographs of arrays of 5 ISU (colour densities

3.2 Pollen sample optimization: Inhibition immunoassay using the allergens released by pollen content as inhibitor

Figure 1B shows the inhibition curves using the three sera and the allergens released by different pollen concentrations as inhibitor in solid phase (ISAC

	Serum 2 ISU	Serum 5 ISU	Serum 15 ISU
<u>Linear part of the curve</u>			
Constant	-38.11	-35.56	-30.21
Slope	45.54	47.24	47.51
R ²	0.94	0.96	0.92
R	0.97	0.98	0.96
Detection limit (pg/mL)	2.3	2.1	1.9
Quantification limit (pg/mL)	2.4	2.1	1.9
<u>Logit regression</u>			
Constant	-4.209	-4.387	-4.088
Slope	0.992	1.019	1.020
R ²	0.80	0.84	0.85
R	0.90	0.92	0.92
<u>Coefficient of Variation (%)</u>			
Ag50 (pg/mL)	10.73	17.74	16.36
	6.9	6.1	5.4

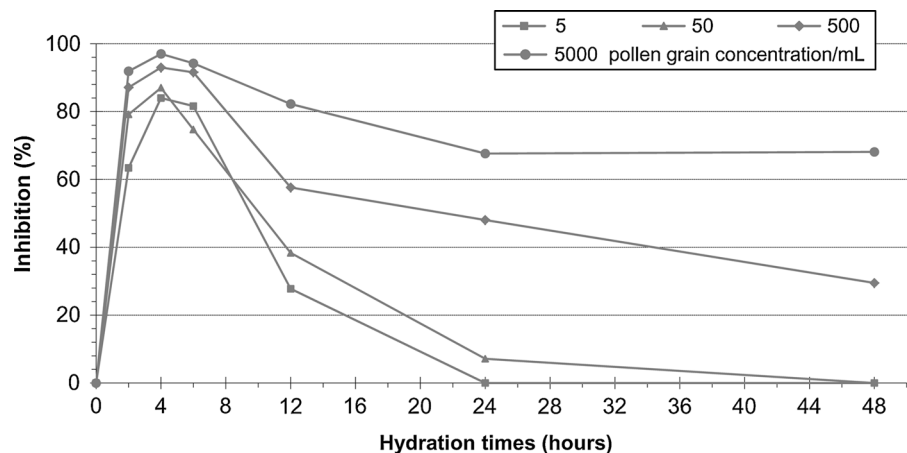
	Serum 2 ISU	Serum 5 ISU	Serum 15 ISU
<u>Linear regression</u>			
Constant	23.13	11.22	5.05
Slope	27.58	24.90	26.31
R ²	0.98	0.97	0.97
R	0.99	0.99	0.98
Detection limit (pollen/mL)	1.0	1.0	1.0
Quantification limit (pollen/mL)	1.0	1.0	1.0
<u>Coefficient of Variation (%)</u>			
Inhibitor concentration	1 pollen grain/mL	15.53	22.13
	5 pollen grain/mL	15.50	22.09
	50 pollen grain/mL	12.60	14.98
	500 pollen grain/mL	0.00	0.77
	5000 pollen grain/mL	0.00	0.00

decrease with increasing percentages of inhibition); Inhibitor assay curves; statistical results

platform). The detection limit and quantification limit were 1 grain of fresh pollen per mL.

The CV values obtained in this assay are shown in Fig. 1B. It can be observed that the greater the amount of inhibitor and serum IgE concentration, the lower was the CV. For instance, while the use of the serum with 5 ISU gave a CV varying from 22.1 % for

Fig. 2 Phl p 1 release from *P. pratense* fresh pollen at different hydration times



1 and 5 pollen grains to 0.8 % for 500 pollen grains, the use of the serum with 15 ISU gave a CV varying from 1.7 % for 1 and 5 grains to 1.4 % for 500 grains.

Figure 2 shows the kinetics of Phl p 1 release from the pollen grains. Results showed that 4 h of hydration was the optimum time to measure the allergens released from pollens.

3.3 Application of Microarrays to the analysis of environmental samples

Figure 3 shows the season pattern of the mean daily airborne Poaceae pollen concentrations and the airborne Phl p 1 during 2009. It also shows the annual pattern of airborne Poaceae pollen during the period 1994–2008. As expected, the pollen pattern during 2009 registered similar dynamics to that observed for the period 1994–2008. The highest pollen concentrations began to be detected at the beginning of April. The peak pollen count in 2009 was observed at June 2 (77 Pollen/m³).

For the airborne allergens, the 11 measurements obtained quite accurately followed the pollen grains pattern. Both parameters showed the highest values on the same days (May 29 and June 2), with 71–77 Pollen/m³ and 8.8–8.9 pg/m³, respectively, as well as a significant decrease on May 30 (24 Pollen/m³; 1.3 pg/m³). However, in some cases (May 10 and 21 and June 8 and 19), the pollen concentration was low while the allergen measurement was high, and in other (April 28) the allergen concentration was zero.

To evaluate all these analyses, we selected the samples from December 30 to be used as a control. The results obtained were zero in both the aerobiological and the immunological tests.

Finally, airborne Poaceae pollen counts and atmospheric Phl p 1 allergenic load were compared in Fig. 4. The Spearman's correlation test was carried out to determine the degree of association between both variables. In this case, a significant correlation was found (0.69; $p < 0.05$).

4 Discussion

This paper presents a novel study for the evaluation of outdoor air quality based on a modification of the specific IgE analysis for individual allergens using a microarray platform. Despite microarrays being a multiplexing concept and this platform including more than 100 different allergenic proteins, the validation of this technology cannot be evaluated using all allergens together, as the high number of possible combinations makes it very difficult to conduct one experiment with such a large quantity of parameters. Thus, we decided to evaluate the method by firstly using the Phl p 1 model.

The study of variability of the three sera used as reference (Fig. 1A) showed their interchangeability. The application of logistic regression established that the IgE levels of the serum do not limit the study and that the detection of Grass Group 1 allergen can be made using human sera with values higher than 2 ISU, since these values are above the detection levels of the curve. Moreover, and according to Martinez et al. (1985), the similar values of Ag50 for all sera further reinforced the fact that the interchangeability of the sera is possible.

It is a common situation that the human serum obtained from patients sensitized to several allergens

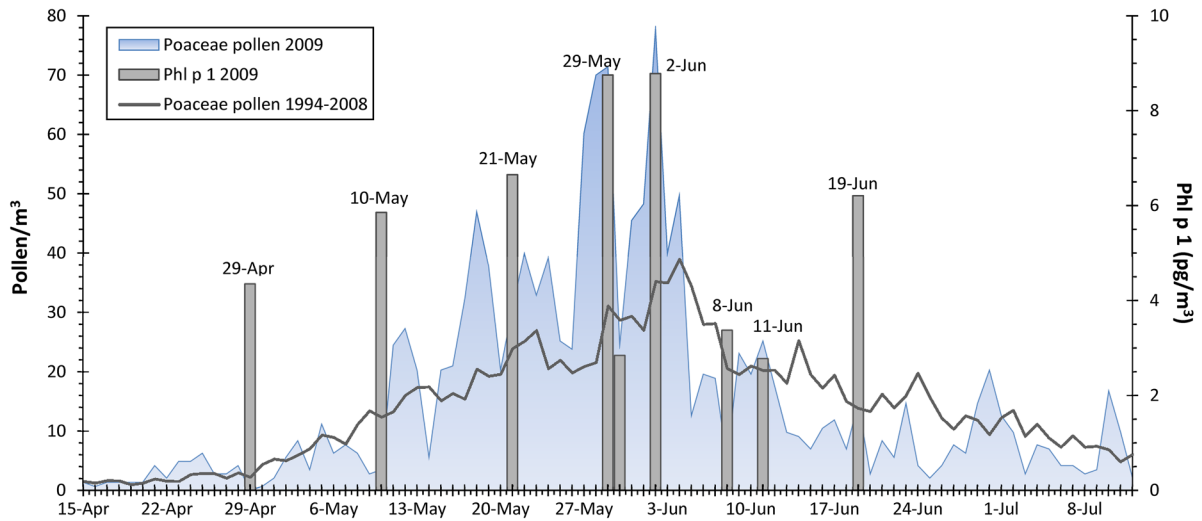


Fig. 3 Comparison of airborne Poaceae pollen (Pollen/m^3) and airborne Phl p 1 allergen (pg/m^3) concentrations analyzed using microarrays technology (Note: there are two measurements

which resulted in $0 \text{ pg}/\text{m}^3$, on 28 April and on 30 December (which was analyzed as a control sample))

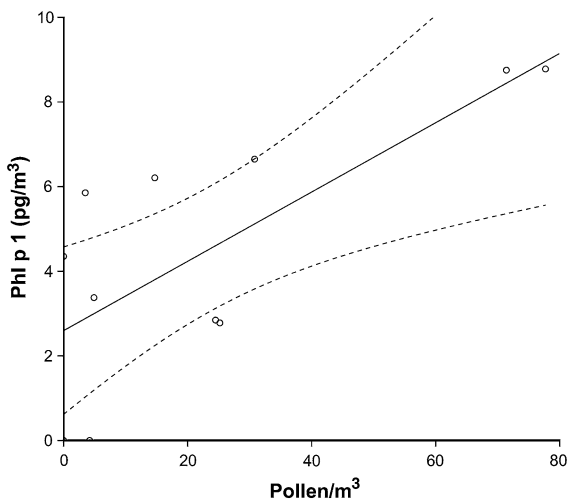


Fig. 4 Poaceae pollen (Pollen/m^3) versus airborne Phl p 1 allergen (pg/m^3) analyzed by microarrays technology

shows different titre for specific IgE to each allergen (Casquete-Román et al. 2009). However, the data obtained with timothy allergens show that the use of only one serum to measure different allergens in the same experiment is viable but must be analyzed in further studies.

Another approach included in this study was a calibration step based on the detection limit of the protein concentrations. It showed that microarrays are

highly sensitive, as demonstrated by a linear detection curve from 1 to 1,000 pg/mL , with correlations higher than 0.96 in all cases (Fig. 1A). The coefficients of variation obtained in this study (10.7–17.7 %) agree with that obtained by Earle et al. (2007), which demonstrated coefficients of variation in the same range using microarrays technology to measure indoor allergens. Therefore, it may be considered as reasonable tool for monitoring outdoor airborne allergen concentrations.

The study by inhibition immunoassay using the allergens released by the pollen as inhibitor (Fig. 1B) showed that protein microarrays are a very sensitive tool, as they are able to detect allergen release from 1 pollen grain (the minimum quantifiable value in Aerobiology techniques). The high sensitivity performance of this platform could be explained by the low amount of allergen bound to the solid phase and the equivalent concentration of antibodies in the human serum needed to work in the optimal antigen–antibody equivalence reaction zone.

This study demonstrates that microarray technology is the most sensitive technique available for the detection of Phl p 1. We were able to quantify between 1.9 and 2.4 pg/mL and the allergens released from 1 pollen grain.

Prior to the detection and quantification of the allergens in the atmosphere, another calibration step was performed. Pollen allergens are inside the pollen

grains, and their release is a prerequisite to trigger allergy (D'Amato et al. 2007). The allergens may be released in a humid environment at different time intervals from a few minutes to hours (Staff et al. 1999; Suárez-Cervera et al. 2003; Vega-Maray et al. 2004). Thus, we observed, in natural conditions, that the maximum release of Group 1 Grass allergen in natural *P. pratense* pollen took place in 4 h (Fig. 2). After this time, the amount of released allergen decreased significantly due to possible exhaustion of allergen contents in the pollen (Holmquist and Vestberg 2003), or the possible incapacity of the pollen grain to expel cytoplasm (Grote et al. 2001) and subsequent degradation of released material. Taking into account this result and the high sensitivity of the method evaluated here, the ability to detect the allergen material released by pollen grains, the concept of the standardization of the sample processing is reinforced, and the period of sample hydration is critical. However, additional studies are needed to better understand this phenomenon.

Finally, to validate the potential use of this methodology, it was decided to examine the relationship between the quantities of grass pollen and Phl p 1 allergen in the air. Assuming that the amounts to measure in aerobiological samples would be small, we postulated that the results could be sufficient for determining whether this technique is valid for use in biological air quality monitoring.

According to our results (Fig. 3), pollen counts and Group 1 grass allergen showed similar dynamics during the main pollination period. The highest values of allergen were reached on the same days as airborne pollen (May 29 and June 2), as was observed by Shäppi et al. (1996), Cabrera et al. (2002), De Linares et al. (2010a) and Rodríguez-Rajo et al. (2011). However, in some days (May 10 and 21 and June 8 and 19), allergen levels were detected when the concentration of airborne pollen was low. This fact was found in other studies and has not yet been clarified (Cabrera et al. 2002; De Linares et al. 2010a; Rodríguez-Rajo et al. 2011). It has been postulated that meteorological factors play an important role in the presence of pollen allergens in the atmosphere. The rupture of pollen grains or allergen release can be influenced by mild temperatures, high humidity or rainfall (Spieksma and Nikkels 1999; De Linares et al. 2010a; Rodríguez-Rajo et al. 2011). However, an allergen may also be transported in PM 2.5 fine particles of released

allergen by pollen, fragments of pollen, grass anthers, other parts of the plants or through orbiculus or starch granules (Emberlin 1995; Knox et al. 1997; Taylor et al. 1994; Suárez-Cervera and Seoane-Camba 2005; Taylor et al. 2007). Even so, Fig. 3 shows that both measurements were gradually increasing until reaching the peak day and then decreased, thus observing good agreement between Phl p 1 allergen and grass pollen concentration. Moreover, the relationship between Poaceae pollen counts and atmospheric allergenic load showed good correlation coefficient *R* and Spearman's *Rho* test.

In conclusion, this study reveals that the application of protein microarrays to monitor the content of allergens in the air could be readily extended to other outdoor allergens included in the microarray panel. Despite one of the possible drawbacks that could have this methodology is the availability of human serum and the current regulations for its use, the characteristics of this technique have two principal advantages: (1) guarantees the allergenic role of the detected proteins on the human health, (2) quantifies several allergens in a single analysis. Consequently, the research could increase further until being able to monitor all airborne allergens that cause allergies and establish a more effective methodology for biological air quality and prevention of respiratory allergy symptoms.

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