

Comparison of extraction methods for Poaceae pollen allergens

Iris Aloisi  · Stefano Del Duca  · Paola De Nuntiis  · Paolo Mandrioli  ·
Delia Fernández-González 

Received: 17 May 2018 / Accepted: 3 October 2018
© Springer Nature B.V. 2018

Abstract While pollen sampling has been standardised in the last decades, allergen extraction from aerobiological samples still needs standardisation. With the aim to identify the most practical, efficient and reproducible method, in this study, protein extraction protocols from Poaceae pollen, i.e. *Lolium perenne*, *Phleum pratense* and *Dactylis glomerata*, were evaluated. Three extraction protocols were selected and compared in terms of protein yield and allergen extraction. The methods were based on the use of: (1) lysis buffer; (2) pollen hydration in PBS buffer followed by sonication; and (3) pollen hydration in PBS buffer. After extraction, protein concentration was measured. Results indicated that the different extraction protocols could extract significantly different protein amounts, being the lysis buffer-based protocol the most efficient in terms of total protein extraction. However, when allergen extraction was compared, no significant differences

were detectable among the different protocols. Results afforded the selection of a suitable extraction protocol for Poaceae pollen allergens. In particular, results suggested a PBS extraction followed by sonication; as it allowed the highest allergen extraction, it might be the most reproducible protocol minimising any possible interference with proteomics and immunological studies when compared to lysis buffer method.

Keywords Comparison of extraction protocol · Poaceae pollen · Poaceae allergens · Protein yield

1 Introduction

Among airborne particles of biological origin, pollen and pollen-derived particles are of great interest as they may cause allergic reactions in both upper and lower respiratory tracts. The detection and monitoring of airborne allergens is generally conducted through a multi-step process, which involves sample collection, sample processing and finally sample analysis (Morris et al. 2014). While pollen monitoring has been standardised in the last decades (Galan et al. 2014), allergen sampling and processing still needs standardisation (Renstrom 2002). Standardisation of both allergen sampling and analysis methods has been a big goal in the last years and, although more effort is necessary, it appears possible to reach a widespread

I. Aloisi · S. Del Duca (✉)
Department of Biological, Geological and Environmental
Sciences, University of Bologna, Via Imerio 42,
40136 Bologna, Italy
e-mail: stefano.delduca@unibo.it

P. De Nuntiis · P. Mandrioli · D. Fernández-González
Institute of Atmospheric Sciences and Climate, ISAC-
CNR, Bologna, Italy

D. Fernández-González
Department of Biodiversity and Environmental
Management, University of León, León, Spain

standardisation of methods (Renstrom 2002; Morris et al. 2014). What is missing is a comparison of different extraction methods of air samples, which can be performed in a number of ways, affecting presumably both allergen elution efficacy and sample stability (Renstrom 2002). The most frequently used extraction methods for airborne pollen-derived samples are based on a lysis buffer extraction (Takahashi et al. 2001; Moreno-Grau et al. 2006; Fernández-González et al. 2011; Plaza et al. 2016; Aloisi et al. 2018) or on pollen hydration into a low-concentrated salt buffer (Schappi et al. 2001; De Linares et al. 2010; Grewling et al. 2016). For this reason, the aim of this work was to evaluate the efficacy and the reproducibility of three protein extraction protocols for small amount of pollen samples. The extraction methods were selected among the most frequently used for airborne allergen sampling: lysis buffer; pollen hydration in PBS buffer and sonication; and pollen hydration in PBS buffer only. For this purpose, small amounts of Poaceae pollen were analysed for both protein and allergens extraction yields. *Lolium perenne*, *Phleum pratense* and *Dactylis glomerata* were selected as they represent a major outdoor seasonal source of airborne allergens and because they play an important role in triggering pollinosis and allergic asthma. The allergenicity of grass pollen is attributed to a small number of proteins that are rapidly released from pollen grains after hydration in the air followed by contact with human mucosa. When pollen comes in contact with a medium, its allergenic content is released in form of single allergen proteins or small granules (0.6–2.5 µm) which can bind to smaller inorganic particles present in the atmosphere, causing allergenic reactions also in the distal parts of the lungs (Suphioglu et al. 1992).

These allergens share similar physicochemical and immunological features, which cause a high incidence of allergenic cross-reactivity on the one hand, and on the other, allows their classification into 14 groups (Weber 2007). The two major allergens of grass pollen are group 1, glycoproteins with a molecular mass of 32–35 kDa, and group 5, ribonucleases with a molecular mass of 28–32 kDa (Weber 2007). Both being major allergens present in atmospheric particles smaller than pollen grains, these were taken into consideration for extraction efficiency. Besides these allergens, also the panallergen profilin was evaluated, as grass pollen profilins seem to be the most potent

pollen-derived inducers of IgE against profilin (Santos and Van Ree 2011).

2 Materials and methods

2.1 Pollen material

The pollen samples of *Dactylis glomerata*, *Lolium perenne* and *Phleum pratense*, grown in a natural field, were collected from 15 anthers of each species in the spring of 2016; weather conditions during all sampling days were calm and dry. At the sampling stage, anthers were closed, in a state of pre-anthesis. The inflorescences were deposited on aluminium foil, in separate chambers, under environmental laboratory conditions (50% RH and 25 °C). The collected pollen was stored frozen at – 20 °C in 1.5 ml vials.

2.2 Protein extraction

According to laboratory standards, before protein quantification, pollen was previously suspended in PBS and serial dilutions were made in order to quantify pollen material. No significant differences were noticed among pollen types and 1 mg of pollen corresponded to ca. 50,000 pollen grains.

Method I Lysis buffer. Extraction was performed as previously reported, with minor modifications (Takahashi et al. 2001; Moreno-Grau et al. 2006; Aloisi et al. 2018). Briefly, pollen was extracted at room temperature for 2 h with phosphate buffer (50 mM pH 7.0) supplemented with 150 mM NaCl, 3 mM EDTA, 0.005% Tween 20 and 125 mM ammonium bicarbonate. The pollen/buffer ratio was constant (10 mg: 120 µl). The extract was separated by centrifugation at 2000×g for 10 min and then stored at –20 °C and the supernatants analysed for protein content.

Method II Hydration in PBS buffer and sonication. Pollen proteins were extracted in PBS for 30 min by continuous agitation followed by 3 min sonication in water bath at 4 °C. Pollen/buffer ratio was constant (10 mg: 120 µl). After centrifugation at 2000×g for 10 min, supernatants and pellet were separated and then supernatants analysed for protein content, whereas pellet was extracted with the lysis buffer as in method I in order to extract proteins left in the pollen grains.

Method III Washing in PBS. Pollen proteins were extracted in PBS for 30 min by continuous agitation. Pollen/buffer ratio was constant (10 mg: 120 µl). After centrifugation at $2000\times g$ for 10 min, supernatants and pellet were separated, then supernatants analysed for protein content, whereas pellet was extracted with the lysis buffer as in method I in order to extract proteins left in the pollen grains.

2.3 Protein quantification and western blotting

The protein content in the supernatants was measured using the dye-binding method of Bradford, with BSA as the reference standard. Proteins (20 µl) were separated by denaturing sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) then electroblotted onto nitrocellulose membranes and incubated overnight at 4 °C with the primary antibodies raised in mouse (Bial Industrial Farmacéutica, Spain). After washing, the membranes were incubated with alkaline-phosphatase-conjugated anti-mouse IgG (1:10,000; Sigma-Aldrich) for 1 h at room temperature. Labelling was detected using the colorimetric substrate AEC (Sigma-Aldrich).

2.4 Statistical analysis

Experiments were performed in triplicate and repeated three times with similar results. Differences within sample sets were determined by analysis of variance (one-way ANOVA), while the overall significances were analysed by a two-way factorial analysis of variance (two-way ANOVA). Mean comparisons were made by applying Tukey's post hoc test, and differences were considered significant at $P < 0.05$. Analyses were performed using GraphPad Prism.

3 Results and discussion

The development of a reproducible and effective method for the extraction of pollen-derived allergens from aerobiological samples can be based on two parameters, the amount of total proteins and allergens extracted. The yield of total proteins extracted with the three methods tested was compared by SDS-PAGE and protein quantification. As shown in Fig. 1A, all three extraction protocols allowed extracting proteins spanning in a broad range of molecular weight.

Separating the same volume of protein extracts allowed preliminary comparisons among the extraction methods.

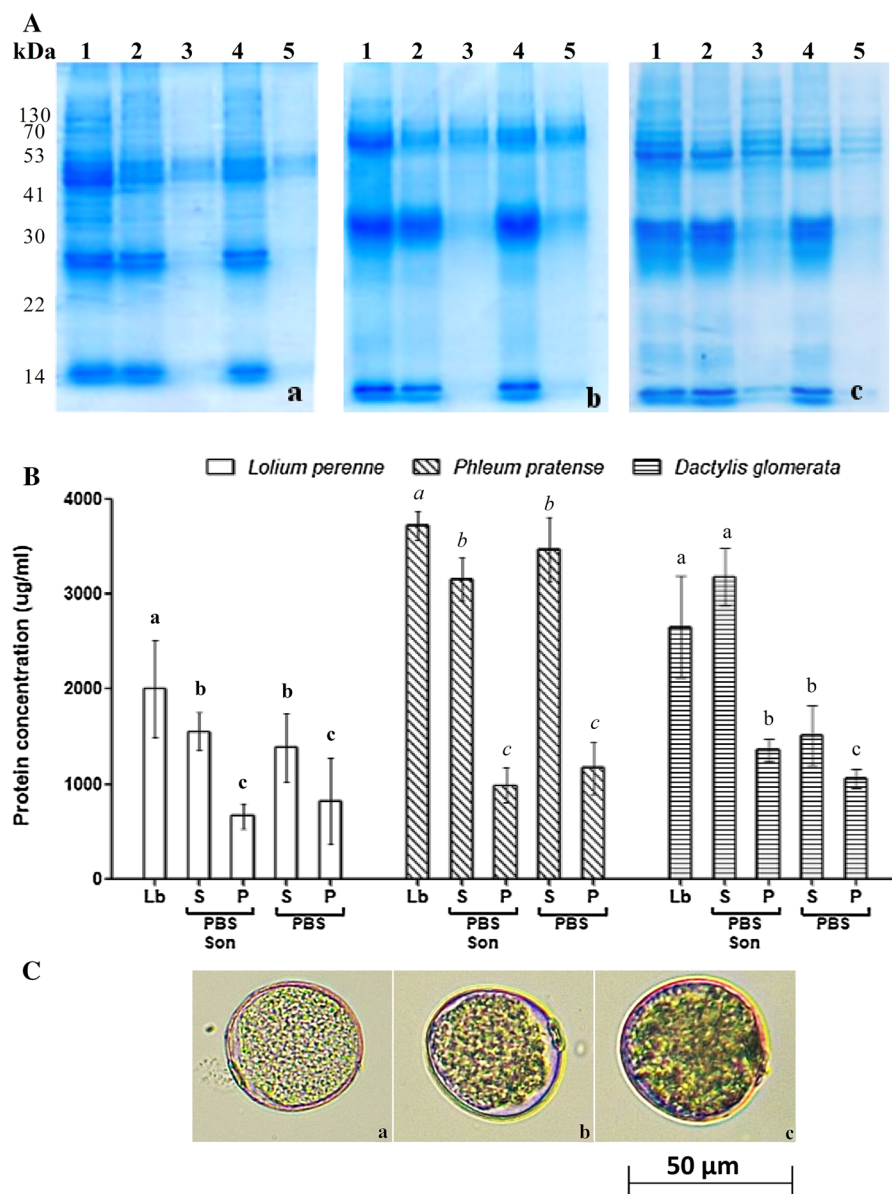
The extraction with lysis buffer (Fig. 1A a–c, lanes 1) allowed a major recovery of total proteins for all three Poaceae pollen as the presence of NaCl, EDTA and Tween as detergent allows extracting and solubilising cell wall and membrane-bound proteins, which are hard to dissolve in water and salt buffers without detergent and/or metal chelating agents. PBS extraction followed by sonication and PBS extractions without sonication (Fig. 1A a–c, lanes 2 and 4) allowed a similar recovery of total proteins even if the pattern of proteins along the lanes showed differences among the three different types of pollens. Preliminary results obtained by SDS-PAGE were confirmed by protein quantification (Fig. 1B). What was worth of noting was the reproducibility of the three extraction methods, but also the pollen-specific yield of total proteins extracted. In fact, while *Dactylis glomerata* and *Phleum pratense* showed comparable protein yields for all the three extraction methods, *Lolium perenne* pollen was characterised by lower protein yields (Fig. 1B, Table 1). This was not due to lower amounts of pollen grains extracted nor to differences in grains diameter, as pollen diameters of all the analysed pollen types were similar (Fig. 1C).

The lower yield of protein extract in *Lolium perenne* when compared to *Phleum pratense* is supported by the literature data. In fact, the protein content of *Lolium perenne* is 15–40% lower when compared to *Phleum pratense* (Jung et al. 2018). Moreover, when the analysis was performed on group 5 allergen, a similar trend was observed; the values of group 5 allergen concentration ranged from 90 µg/ml (*Lolium perenne*) to 295 µg/ml (*Phleum pratense*).

The amount of Poaceae pollen allergens, extracted with the three methods, was compared by western blotting. Results are shown in Fig. 2. The selected primary antibodies were able to recognise the major Poaceae allergens of the selected pollen, i.e. *Lolium perenne*, *Phleum pratense* and *Dactylis glomerata*. What was worth noticing was a higher immuno-recognition signal in the samples derived from the PBS extraction followed by sonication (Fig. 2, lanes 2) when compared to the other extraction methods. This was clear for group 5 and profilin allergens, but also for group 1 allergen, even if the difference for this allergen was less evident between method I (extraction

Fig. 1 Protein yield of three protein extraction methods. **A** SDS-PAGE of Poaceae proteins extracted by three different methods stained with Coomassie blue. Protein patterns of *Lolium perenne* (a), *Phleum pratense* (b) and *Dactylis glomerata* (c). Lane 1: extraction with lysis buffer; lanes 2 (supernatant) and 3 (pellet) from PBS extractions followed by sonication; lanes 4 (supernatant) and 5 (pellet) from PBS extractions.

B Protein concentration ($\mu\text{g}/\text{ml}$). Values are mean $\pm$ SD of three replicates analysed in triplicate. **C** Pollen grains of *Lolium perenne* (a), *Phleum pratense* (b) and *Dactylis glomerata* (c) at optical microscope, $40\times$ magnitude



with lysis buffer, Fig. 2, lanes 1) and method II (PBS extraction followed by sonication, Fig. 2, lanes 2), especially for *Phleum pratense*.

4 Conclusions

All the extraction methods investigated in this research were demonstrated to be highly reproducible; however, it was clear that the extraction of pollen allergens in PBS buffer followed by sonication was

more efficient, at least for the three pollen types selected in this study. Therefrom, we suggest this extraction method for small amounts of pollen samples, like daily airborne samples. Moreover, according to easiness, velocity, efficacy and reproducibility of the method II in terms of proteins and allergens extraction, we suggest that this method could fit also for high amount of pollen as reference protocol allowing comparing data from different species and laboratories involved in pollen allergens research.

Table 1 The statistical analysis performed in order to compare the three extraction methods

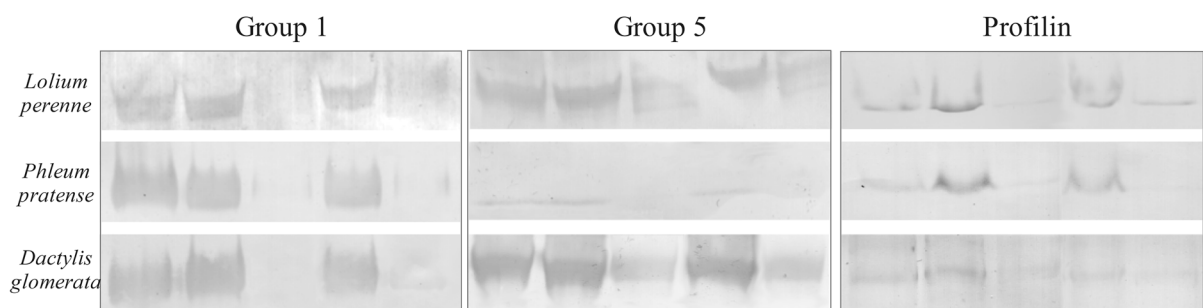
	Mean diff	Adjusted <i>P</i> value	Summary
<i>Multiple comparison analysis performed by two-way ANOVA, with Tukey's post-test</i>			
Lysis buffer			
<i>Lolium perenne</i> versus <i>Phleum pratense</i>	− 1713	0.0015	**
<i>Lolium perenne</i> versus <i>Dactylis glomerata</i>	− 649	0.3179	ns
<i>Phleum pratense</i> versus <i>Dactylis glomerata</i>	1064	0.0558	ns
Son/PBS Soluble			
<i>Lolium perenne</i> versus <i>Phleum pratense</i>	− 1595	0.003	**
<i>Lolium perenne</i> versus <i>Dactylis glomerata</i>	− 1621	0.0026	**
<i>Phleum pratense</i> versus <i>Dactylis glomerata</i>	− 26.12	0.9981	ns
Son/PBS pellet			
<i>Lolium perenne</i> versus <i>Phleum pratense</i>	− 323.7	0.7451	ns
<i>Lolium perenne</i> versus <i>Dactylis glomerata</i>	− 693.5	0.2723	ns
<i>Phleum pratense</i> versus <i>Dactylis glomerata</i>	− 369.8	0.6819	ns
PBS soluble			
<i>Lolium perenne</i> versus <i>Phleum pratense</i>	− 2091	0.0001	***
<i>Lolium perenne</i> versus <i>Dactylis glomerata</i>	− 128.1	0.9546	ns
<i>Phleum pratense</i> versus <i>Dactylis glomerata</i>	1963	0.0003	***
PBS pellet			
<i>Lolium perenne</i> versus <i>Phleum pratense</i>	− 349.2	0.7104	ns
<i>Lolium perenne</i> versus <i>Dactylis glomerata</i>	− 238.7	0.8514	ns
<i>Phleum pratense</i> versus <i>Dactylis glomerata</i>	110.4	0.966	ns
Source of variation	% Of total variation	<i>P</i> value	<i>P</i> value summary
<i>Source of variation and interaction analysis performed by two-way ANOVA</i>			
Interaction	14.26	0.0078	**
Extraction method	49.13	< 0.0001	****
Pollen type	20.49	< 0.0001	****

* differences were considered significant at $P < 0.05$

** differences were considered significant at $P < 0.01$

*** differences were considered significant at $P < 0.001$

**** differences were considered significant at $P < 0.0001$

**Fig. 2** Allergens extraction using three extraction methods. Proteins (20 μ l) were separated by SDS-PAGE and electroblotted onto nitrocellulose membranes. Membranes were incubated with group 1, group 5 and profilin primary antibodies

Acknowledgements We are very thankful to Juan Asturias for providing pollen samples and primary antibodies. This study was partially supported by AERORAIN project (Ministry of Economy and Competitiveness, Grant CGL2014-52556-R, co-financed with FEDER funds).

References

- Aloisi, I., Del Duca, S., De Nuntis, P., Vega Maray, A. M., Mandrioli, P., Gutiérrez, P., et al. (2018). Behavior of profilins in the atmosphere and in vitro, and their

- relationship with the performance of airborne pollen. *Atmospheric Environment*, 178, 231–241. <https://doi.org/10.1016/j.atmosenv.2018.01.051>.
- De Linares, C., Diaz de la Guardia, C., Nieto Lugilde, D., & Alba, F. (2010). Airborne study of grass allergen (Lol p 1) in different-sized particles. *International Archives of Allergy and Immunology*, 152(1), 49–57. <https://doi.org/10.1159/000260083>.
- Fernández-González, D., Rodríguez Rajo, F., González Parrado, Z., Valencia Barrera, R. M., Jato, V., & Grau Moreno, S. (2011). Differences in atmospheric emissions of Poaceae pollen and Lol p 1 allergen. *Aerobiologia*, 27, 301–309. <https://doi.org/10.1007/s10453-011-9199-x>.
- Galan, C., Smith, M., Thibaudon, M., Frenguelli, G., Oteros, J., Gehrig, R., et al. (2014). Pollen monitoring: Minimum requirements and reproducibility of analysis. *Aerobiologia*, 30(4), 385–395. <https://doi.org/10.1007/s10453-014-9335-5>.
- Grewling, L., Bogawski, P., Jenerowicz, D., Czarnecka-Operacz, M., Sikoparija, B., Skjoth, C. A., et al. (2016). Mesoscale atmospheric transport of ragweed pollen allergens from infected to uninfected areas. *International Journal of Biometeorology*, 60(10), 1493–1500. <https://doi.org/10.1007/s00484-016-1139-6>.
- Jung, S., Estrella, N., Pfaffl, M. W., Hartmann, S., Handelshausen, E., & Menzel, A. (2018). Grass pollen production and group V allergen content of agriculturally relevant species and cultivars. *PLoS ONE*, 13(3), e0193958. <https://doi.org/10.1371/journal.pone.0193958>.
- Moreno-Grau, S., Elvira-Rendueles, B., Moreno, J., Garcia-Sanchez, A., Vergara, N., Asturias, J. A., et al. (2006). Correlation between *Olea europaea* and *Parietaria judaica* pollen counts and quantification of their major allergens Ole e 1 and Par j 1-Par j 2. *Annals of Allergy, Asthma & Immunology*, 96(6), 858–864. [https://doi.org/10.1016/S1081-1206\(10\)61350-6](https://doi.org/10.1016/S1081-1206(10)61350-6).
- Morris, D. R. P., Fatisson, J., Olsson, A. L. J., Tufenkji, N., & Ferro, A. R. (2014). Real-time monitoring of airborne cat allergen using a QCM-based immunosensor. *Sensors and Actuators B: Chemical*, 190, 851–857. <https://doi.org/10.1016/j.snb.2013.09.061>.
- Plaza, M. P., Alcázar, P., Hernández-Ceballos, M. A., & Galán, C. (2016). Mismatch in aeroallergens and airborne grass pollen concentrations. *Atmospheric Environment*, 144, 361–369. <https://doi.org/10.1016/j.atmosenv.2016.09.008>.
- Renstrom, A. (2002). Exposure to airborne allergens: A review of sampling methods. *Journal of Environmental Monitoring*, 4(5), 619–622.
- Santos, A., & Van Ree, R. (2011). Profilins: mimickers of allergy or relevant allergens? *International Archives of Allergy and Immunology*, 155(3), 191–204. <https://doi.org/10.1159/000321178>.
- Schappi, G. F., Taylor, P. E., Pain, M. C., Cameron, P. A., Dent, A. W., Staff, I. A., et al. (2001). Concentrations of major grass group 5 allergens in pollen grains and atmospheric particles: Implications for hay fever and allergic asthma sufferers sensitized to grass pollen allergens. *Clinical and Experimental Allergy*, 29(5), 633–641.
- Suphioglu, C., Singh, M. B., Taylor, P., Bellomo, R., Holmes, P., Puy, R., et al. (1992). Mechanism of grass-pollen-induced asthma. *Lancet*, 339(8793), 569–572. [https://doi.org/10.1016/0140-6736\(92\)90864-Y](https://doi.org/10.1016/0140-6736(92)90864-Y).
- Takahashi, Y., Ohashi, T., Nagoya, T., Sakaguchi, M., Yasueda, H., & Nitta, H. (2001). Possibility of real-time measurement of an airborne *Cryptomeria japonica* pollen allergen based on the principle of surface plasmon resonance. *Aerobiologia*, 17, 313–318. <https://doi.org/10.1023/A:1013002001583>.
- Weber, R. W. (2007). Patterns of pollen cross-reactivity of pollen allergens: impact on allergen immunotherapy. *Annals of Allergy, Asthma & Immunology*, 99, 203–211. [https://doi.org/10.1016/S1081-1206\(10\)60654-0](https://doi.org/10.1016/S1081-1206(10)60654-0).