Allergenic potency of birch pollen

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SUMMARY
The number of people sensitized to the major birch pollen allergen Bet v 1 in industrialized countries is vast and still rising. The purpose of this study was to develop a method with sensitivity high enough to measure the Bet v 1 content of a few birch pollen grains without the interference of environmental factors such as diesel particles, ozone level, humidity, temperature and precipitation. Grains were collected from catkins at two locations in Sweden over a period of 5 years. Allergens were extracted over polytetrafluoroethylene (PTFE) filters and Bet v 1 quantification was made with a luminescence immunoassay. The average content of Bet v 1 was 3.6 ± 0.6 pg per pollen grain for samples collected in three different pollination seasons.

This is the first in a series of controlled experiments on the release of the major allergen Bet v 1 from birch pollen grains.

PRACTICAL IMPLICATIONS
An immunoassay with the ability to detect the content of Bet v 1 in a single or a few birch pollen grains was developed. It is now possible to study pollen potency using grains with the same environmental history.

KEYWORDS
Bet v 1, *Betula pendula*, luminescence, allergy, immunoassay

1 INTRODUCTION

The prevalence of asthma has increased for the past 40 years and studies show that 5-30% of the inhabitants in industrialized countries suffer from allergy with symptoms such as asthma and rhinitis. In order to facilitate preventive health measures, national surveys of pollen counts in ambient air are often provided, using Burkard pollen and spore traps. While exposure to allergens is roughly correlated with pollen counts, large deviations are found on a day to day basis (Sofiev and Bergmann, 2013). The latter indicates that large amounts of allergens may be present in ambient air even if the pollen count is low (Buters et al. 2010). To accurately predict the severity of the allergy symptoms, the pollen counts must therefore be supplemented by additional information to reflect the bioavailability of allergens in the outdoor and indoor environment. Furthermore, the release of allergens from pollen grains (the pollen potency) has been shown to be influenced by environmental factors, such as diesel particles (Behrendt and Becker, 2001), ozone (Beck et al. 2013), humidity (Taylor et al. 2004) temperature (Beck et al. 2013) and precipitation (Schäppi et al. 1997).

The general aim of this study is the development of a method with enough sensitivity and reliability to study the content of Bet v 1 in a few pollen grains. The specific objectives are to present some initial results and make comparisons with previous studies that have used other sampling and extraction methods. A frequently used method of collecting pollen and allergen is by high-volume cascade impactor, that sucks airborne pollen onto filters that are later analyzed for Bet v 1 (Schäppi et al. 1997; Jochner et al. 2015). The weakness of this method is that these airborne grains probably have been exposed to different environmental conditions and could vary in Bet v 1 content. Another common method of collecting birch pollen for later analysis of Bet v 1 is by bringing catkins indoors, drying them and collecting the released pollen followed by liquid extraction from several million grains (Beck et al. 2013; Taylor et al. 2004). This method should give a reliable mean as pg Bet v 1 per grain but is weak in the sense of controllability of the effecting variables on the collected grains e.g. temperature, level of maturity and humidity. Environmental conditions affect the amount of released Bet v 1 and by making extract of large quantities of pollen an average amount is obtained, whereas each individual grain could be different.

The present study is a contribution to the understanding of pollen potency using a method based on previous work by Holmquist and Vesterberg (1999), enhanced to accurately measure the release of Bet v1 from one or a few pollen grains. Filters with 1-7 *Betula pendula* pollen grains each were analyzed for Bet v 1 in this initial study.

2 MATERIALS/METHODS

Pollen from silver birch, *Betula pendula*, was gathered directly from flowering catkins still attached to the trees. Samples were collected at two locations, Gävle and Sandviken, Sweden, at four different dates. Sterile polypropene test tubes were used to slide over the catkins and then simulating a light breeze by gently wagging the test tubes back and forth. Collected pollen were immediately placed in a freezer at -35°C and kept frozen until analysis. Pollen grains, 1-7 pieces, were placed on polytetrafluoroethylene (PTFE) filters, floating on Tris-buffered saline (TBS) in 6-well multidish polystyrene vials. Hypodermic needles were used for the transfer of pollen (monitored in a stereo phase contrast microscope 40x-100x magnification) from the polypropene test tubes to the filters. The positions of the grains on the filters were noted.

The samples were then incubated and analyzed for the major birch allergen Bet v 1, using a newly developed method based on the direct on sampling filter in solution method (DOSIS) Holmquist and Vesterberg (1999). The method for analysis is an immunoassay where the allergens are bound to the PTFE-filter. Quantification is made with alkaline phosphatase (AP) conjugated polyclonal antibodies (swine anti rabbit/AP, DAKO) reacting with the substrate Lumi-phos Plus and creating a luminescent product that is measured with a luminometer, (Luminoskan, Labsystems Oy). The luminescence signal is about three times as strong and the background noise is greatly reduced, compared to the DOSIS method. (The detailed protocol will be submitted elsewhere) Polyclonal antibodies (rabbit anti Bet v 1) were kindly donated by ALK, Denmark.

After analysis the filters were stained with BCIP/NBT reagent for 1 hour, making the allergen dark blue and visible (Acevedo et al. 1998). The filters were then scanned with a Canon LiDE 210 scanner with 4800 dpi resolution. Only data obtained from filters with the correct amount of grains and the correct positions, as determined before and after analysis, were kept for statistical processing.

Calibration was made against natural Bet v 1 proteins (Indoor Biotechnologies), extracted from birch pollen. The calibration curve is highly linear, indicating a strong precision in the method of analysis (Fig. 1). The detection limit is calculated to be 8.5 RLU, using the mean of the background noise (6.1 RLU, n = 12) +2 standard deviations (SD = 1.2). All samples below this signal level are excluded from the statistical analysis.

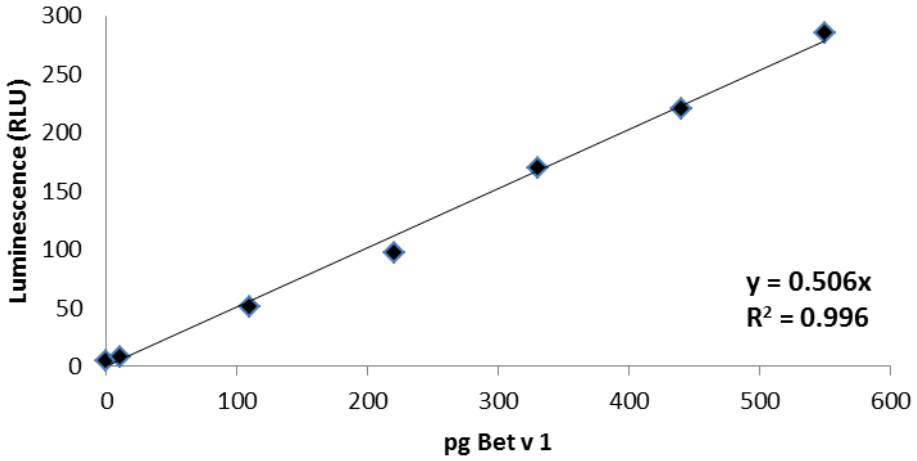

Figure 1. The luminescence in relative light units (RLU) plotted against the mass of Bet v 1 added to 6 different 1.2 µm pore size PTFE filters. Even at low concentration the calibration curve with natural Bet v 1 extracted from birch pollen, show a linear dose dependent signal.

3 RESULTS

With an ELISA type Immunoassay modified to improve sensitivity birch pollen grains were analyzed and preliminary results showed only slight variations among grains (Fig. 2a). The variation among samples (A, B, C and D) did not differ significantly (One-way ANOVA $p = 0.06$) and all the samples were pooled (Fig. 2b). The pooled data was normally distributed (passed a Shapiro-Wilk test for normality with $p = 0.54 > 0.05$) with $\mu = 3.6 \pm 1.0$ (mean ± SD) pg Bet v 1 per grain (n = 15), with a range of 1.7–5.2 pg and the range at the 95% confidence level for the mean is 3.6 ± 0.6 pg.

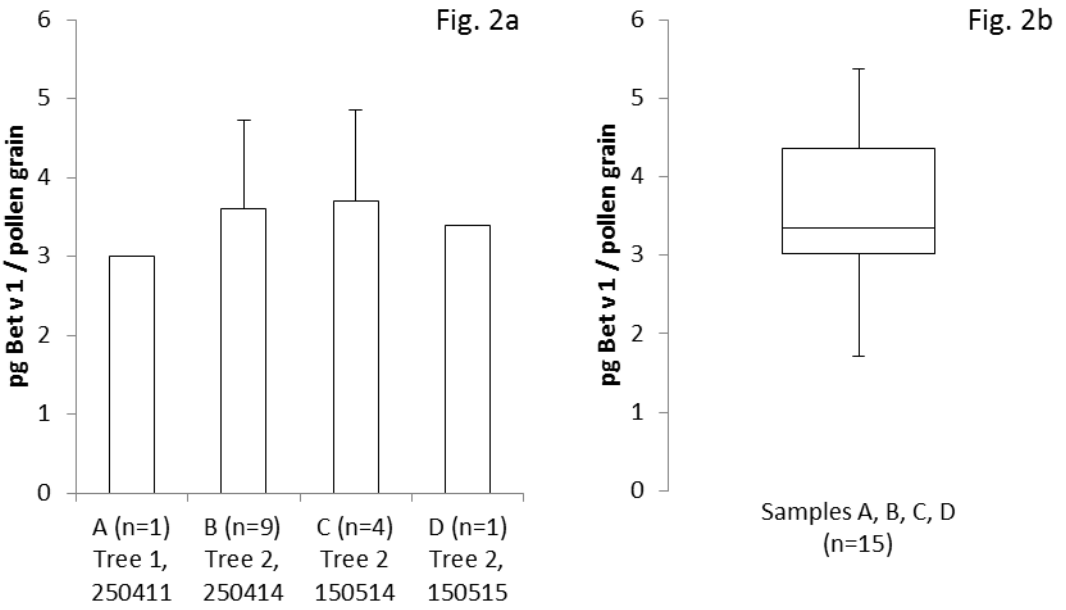

Figure 2a) Pollen potency expressed as pg Bet v 1 per pollen grain in four samples collected from two different trees at four different dates. The bars show mean ± SD given from analysis of PTFE filters, prepared with 1-7 pollen grains per filter. Tree 1 (sample A) is located in Gävle and tree 2 (sample B, C and D) is located in Sandviken. Figure 2b) shows pg Bet v 1/pollen grain when all samples were pooled (n = 15). There were no significant difference in variation among samples ($p = 0.06$).

4 DISCUSSION

There is a need for a method of analyzing pollen potency of single pollen grains. The method should enable the study of grains collected from the same catkin which have been exposed to the same environmental conditions. There are several known environmental variables that interact with pollen, such as diesel particles (Behrendt and Becker, 2001), SO_2 (Behrendt and Becker, 2001), ozone (Beck et al. 2013), humidity (Taylor et al. 2004), temperature (Beck et al. 2013) and precipitation (Schäppi et al. 1997). Pollen affected by the same environmental conditions are ideal to use when controlled experiments are conducted, changing only one variable at a time. The DOSIS immunoassay (Holmquist and Vesterberg, 1999) analysis of Bet v 1 on PTFE filters is greatly enhanced by reducing the background noise to the extent that the signal from the content of Bet v 1 in just one pollen grain was detectable.

Presented results are coherent with most previous studies (Table 1), which report Bet v 1 content at a range from 0.4 to 12.5 pg/pollen. One study shows deviant values, 107.5-203.3 pg Bet v 1/grain which is the calculated results from three different *Betula Pendula* accessions: 'Youngii', 'Fastigiata' and 'Tristis' (Schenk et al 2011).

Table 1. Other authors results as min-max pg Bet v 1 per birch pollen grain, using different methods and presenting data in dissimilar units. Method 1is the use of a Burkard trap for pollen count and a cascade impactor for airborne allergen collection and method 2 is extract of at least 1 mg pollen. The pollen potency presented by Beck (2013) and Schenk (2011) are calculated from the estimation of birch pollen mass of 7.85 ng/grain. Data presented by Taylor (2004) shows that 4 pg Bet v 1/grain is water soluble, 2 pg is bound to the grain and in total every grain contain 6 pg Bet v 1.

Author	Method	Range (pg)	Units presented in reference
Jochner et al 2015	1	1.1-3.7	pg Bet v 1/grain
Buters et al 2012	1	0.6-8,8	pg Bet v 1/grain
Buters et al 2010	1	0.8-8.4	pg Bet v 1/grain
Buters 2013	1	0.5-12.5	pg Bet v 1/grain
Taylor et al 2004	2	4+2=6	pg Bet v 1/grain
Beck et al 2013	2	0.4-11.0	Estimations from figure.
			500-14000 ng Bet v 1/10 mg pollen
Schenk et al 2011	2	107.5-203.3	µg Bet v 1/mg pollen

A large variation of Bet v 1 content in pollen is frequently reported. Buters (2010) explain this variation as different levels of expression of Bet v 1 in the pollen grains during ripening. The expression of Bet v 1 starts about 6 days before the pollination peak and the content in the grains increase by level of maturity. Meanwhile, the local temperature and humidity determines the opening of the catkins, which release the grains to the ambient air. The degree of maturity at the time of release is the decisive factor, explaining the variation between grains. In the same study, data is presented from grains collected in 2007, showing a concentration of 2.97 ± 12.13 pg Bet v 1/pollen. The standard deviation in this case is vast in comparison with the mean and indicates that the data it is not normally distributed (Limpert et al 2001). This frequently occurring erroneous use of parametric statistics assuming normally distributed data but applied to log-normally distributed data is exemplified in the Appendix.

There are several studies showing a discrepancy between pollen concentration and detectable Bet v 1 in ambient air (D`Amato et al. 2007; Schäppi et al. 1999; Rantio-Lehtimäki et al. 1994). One possible explanation could be the degree of ripening, as previously mentioned, and another possible contributor to the variation could be pollen spiked with allergen from other grains. Particles in general agglomerate with other particles and frequently occurring particles in the air during the period of pollination are birch pollen. Pollen grains could release some particulate allergenic starch granules when exposed to light rain (Schäppi et al. 1999) or high humidity (Taylor et al. 2004). If this happens when grains are airborne we speculate that these granules could attach to other birch pollen grains. This would cause some pollen grains to be spiked with allergens. Collecting pollen using Burkard spore traps or impactor based methods probably also collects grains that are affected by long range transport and thereby some pollen could be empty, some spiked or anything in between.

In contrast to other methods that collect large amounts of pollen grains, the enhanced DOSIS method offers the possibility to compare allergen release from a few grains. If these grains are

collected from the same catkin and next to each other, environmental influence ought to be similar. The risk of reading a signal from something other than whole birch pollen, such as a broken pollen grain or a fragment of a catkin, is reduced as the number and position of the grains are checked before and after analysis. The combination of good controllability of the grains and the high sensitivity of the immunoassay makes this method a promising candidate for detecting the difference in Bet v 1 content in pollen grains.

5 CONCLUSIONS

Comparisons between single or a few birch pollen grains with respect to Bet v 1 content is now possible. For pollen grains collected over a 5 year period at two locations, the Bet v 1 content is determined to 3.6 ± 0.6 pg Bet v 1 per grain, at 95% confidence level. Our research on Bet v 1 and pollen potency will continue with a larger set of locations and trees over several years to support these initial findings. The improved analysis method may also facilitate detailed controlled experiments (in progress) of the Bet v 1 expression during the ripening process and also the possibility of allergen attachment onto other pollen grains.

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Appendix

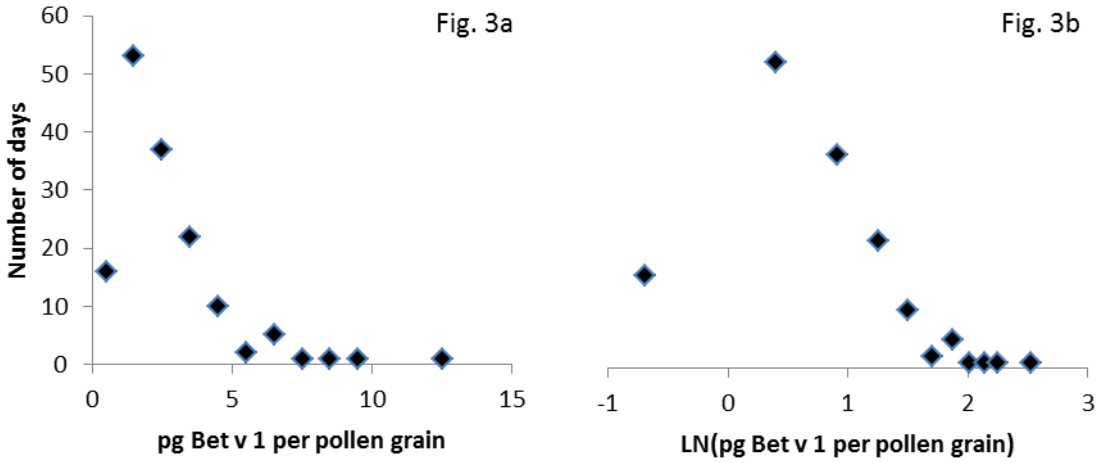

Figure 3a) Binned data of pg Bet v 1/pollen grain in measurements performed 2007-2011 as extracted from Figure 2a in Buters (2013). Figure 3b) The same data plotted with a logarithmic x-axis.

As a clue to the large standard deviations for the pollen potency that are commonly reported (Limpert et al. 2001; Schäppi et al. 1997; Buters et al. 2010), we show the compounded data collected over four years as presented by Buters (2013) in figure 3. Since pollen potency obviously cannot be below zero, it is clear that the data given in figure 3a is not normally distributed. Plotting the same data, but using the natural logarithm of pollen potency, in figure 3b suggests that the data may be closer to a lognormal distribution. (Unfortunately, we only have access to the binned data so the left part of the almost normal curve in figure 3b cannot be reproduced.) Testing for normality using SPSS (IBM 2013), we find that both curves are rejected in a Shapiro-Wilke test for normality. However, in a normal Q-Q plot the logarithmic data in figure 3b closely follow the line whereas the original data in figure 3a clearly deviates.
Using parametric statistics assuming normally distributed data and applying them to the data in figure 3a, we find the average pollen potency to be $\mu = 2.6$ pg Bet v 1 with the 95.5% confidence limits for μ ranging from -1.1 to 6.2 pg. The lower limit falls on the wrong side of zero and we can conclude that the data is erroneously represented. On the other hand, applying the same parametric statistics to the data in figure 3b (that appears to be approximately normally distributed) and transforming back, we find the average pollen potency to be $\mu^* = 2.1$ pg Bet v 1 with the 95.5% confidence limits for μ^* ranging from 1.8 to 2.3 pg. These latter estimates clearly represent the data better and do not result in unphysical predictions.