

Size Determination of Pollens Using Gravitational and Sedimentation Field-Flow Fractionation

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Pollens are known to be an allergen. They penetrate human respiratory system, triggering a type of seasonal allergic rhinitis called pollen allergy (hay fever). The purpose of this study is to test two field-flow fractionation (FFF) techniques, gravitational FFF (GrFFF) and sedimentation FFF (SdFFF), for their applicability to size-characterization of micron-sized pollens. Both GrFFF and SdFFF are elution techniques, providing sequential elution of particles based on size. They allow the size distribution as well as the mean size of the sample to be determined from the elution time. In this study, GrFFF and SdFFF were used to determine the size distribution of Paper Mulberry and Bermuda Grass pollens. For the Paper Mulberry pollen, the mean size obtained by GrFFF is 12.7 μm , and agrees rather well with the OM data with the relative error of 8.0%. For the Bermuda Grass pollen, the mean size obtained by GrFFF is 32.6 μm with the relative error of 12.3%. The mean sizes determined by SdFFF are 12.4 (relative error = 10.1%) and 27.1 μm (relative error = 5.2%) for the Paper Mulberry and the Bermuda Grass pollen, respectively. Although SdFFF tends to yield more accurate size distribution due to lower band broadening under the field strength higher than 1 G, the sizes determined by GrFFF were not significantly different from those by SdFFF.

Key Words : Gravitational field-flow fractionation (GrFFF), Pollen, Size-characterization

Introduction

Pollen is one of the most common causes of human allergic reactions.¹⁻³ Tiny pollen grains are released from various plants (trees, weeds, and grasses), and enter human respiratory systems, triggering a type of seasonal allergic rhinitis called pollen allergy, better known as "hay fever". Although many of the foods, medicines, or animals that cause allergies can be avoided, it is not easy to avoid pollens. Even staying indoors may not help when the pollen count is high.

Since it was first reported,³ the pollen allergy has been studied by many researchers. It has been reported that the size distributions of pollens are usually broad, and the size distribution is one of important factors to the allergic symptoms.¹ Approximately 20% of population in industrialized countries suffers from IgE-mediated (Type I) allergic diseases such as bronchial asthma, conjunctivitis, and rhinitis.⁴ In Republic of Korea, about 30% of adult showed positive reaction to skin-test for pollen allergy.²

Field-flow fractionation (FFF) is a family of separation techniques which have been demonstrated to be able to separate and characterize high molecular weight species in a size range spanning five orders of magnitude, from macromolecules to micron-sized particles.⁵⁻⁷ Among the members of FFF, gravitational field-flow fractionation (GrFFF) is a technique that has been shown to be suitable for quick separation and characterization of particles of about 1-100 μm in size.⁸ Like other FFF members, GrFFF is a chromatography-like separation technique that uses an external field

(or gradient). In GrFFF the external field is the earth's gravity which is applied perpendicularly to a thin (about 100-200 μm thick), empty channel having rectangular cross-section. There is no packing material (stationary phase) inside the channel and the sample is swept down the channel by the carrier liquid. Particles are driven towards the channel outlet at different velocities depending on their sizes. GrFFF has been employed for separation of various particulate materials including red blood cells,⁹⁻¹¹ parasites,^{12,13} wine yeast,^{14,15} wheat starch,^{16,17} inorganic particles,¹⁸⁻²¹ and particles of biological interest.²²⁻²⁴

The aim of this study is to investigate the applicability of GrFFF for separation and the size-characterization of pollens, and then to develop a GrFFF method for size-determine of pollens.

Theory

In an ideal steric mode of GrFFF, where there is no surface interactions, no friction, no flow rate-induced lift forces, the retention ratio, which is defined by the ratio of the retention time of the carrier (t^o) to that of the sample (t_r), R is given by¹⁸

$$R = \frac{t^o}{t_r} = \frac{3d}{w} \quad (1)$$

, where d is the particle diameter and w is the thickness of the GrFFF channel. According to eqn. (1), R is directly proportional to the particle diameter, and thus GrFFF provides size-based separation.

In real world, R could be either larger or smaller than $3d/w$ depending upon the experimental conditions (such as the particle density and diameter, flow rate, and the chemical composition of the carrier, etc.), and is expressed by²⁵

$$R = \frac{3\gamma d}{w} \quad (2)$$

Here γ is a "correction factor" adopted to take the dependence of R on experimental conditions into account. In an ideal case, where the particles are moved only by the flow stream at the distance of one particle radius away from the wall, the value of γ is 1, and eqn 2 becomes Eq. (1). Unfortunately γ has not yet been completely understood, and thus the size-analysis by GrFFF requires a calibration.²⁶

For GrFFF calibration, a series of standards (with known diameters) are injected, and from their observed retention times (t_r), a plot of $\log t_r$ vs. $\log d$ is obtained. Assuming γ is a constant, this plot is expected to be linear. A first order least square fitting to this plot results in a calibration plot which is generally expressed by²⁷

$$\log t_r = -S_d \log d + \log t_{r1} \quad (3)$$

, where S_d is diameter-based selectivity, which is typically around 0.7-0.8, and t_{r1} is a constant equal to the extrapolated value of retention time for particles of unit diameter. By using the calibration parameters S_d and t_{r1} , mass-based particle size distribution, $m(d)$, which gives the relative mass as a function of diameter, can be obtained by²⁸

$$m(d) = c(t_r) \dot{V} \left| \frac{dt_r}{dd} \right| = c(t_r) \dot{V} S_d t_{r1} \left(\frac{t_r}{t_{r1}} \right)^{(S_d+1)/S_d} \quad (4)$$

, where $c(t_r)$ is the detector signal at retention time t_r , and \dot{V} is the volumetric flow rate. In Eq. (3), if the signal is divided by d^2 , the mass distribution is converted to the number-based size distribution.²⁸ Ideally, the densities of the standard and the sample must be same because, if not, γ of the standard might be different from that of the sample, resulting in inaccurate size measurement.

Experimental Section

The pollens used in this study were Paper Mulberry and Bermuda Grass pollen purchased from Duke Scientific Corp. (California, USA). The Paper Mulberry pollen has the nominal size range of 11-17 μm and the Bermuda Grass pollen 21-29 μm . When dry pollens were swelled in water, they are in spherical shape as confirmed by optical microscopy. Their nominal size pertains to the hydrated spherical condition.

The polystyrene latex standards were also purchased from Duke Scientific Corp., and have nominal diameters of 4.5 (with the coefficient of variation (CV) of 20.0%), 14.9 (with CV of 14.8%), 21.4 (with CV of 15.0%), and 45.6 μm (with CV of 14.5%) and the density of 1.05 g/mL.

The GrFFF channel dimensions were 2 cm in breadth (b), 51 cm in length (L), and 254 μm in thickness (w). GrFFF system was equipped with a Gilson Miniplus 3 Peristaltic

Pump (Gilson Medical Electronics, Middleton, WI, USA) and a Young-Lin M720 UV/VIS detector operating at the wavelength fixed at 254 nm (Young-Lin Science, An-Yang, Korea). The void volume (V^0) was measured to be 2.8 mL from the retention volume of 0.5% acetone (v/v).

The Sedimentation FFF (SdFFF) channel is 90 cm in tip-to-tip length, 1.5 cm in breadth, and 0.019 cm in thickness. The rotor radius is 15.1 cm. The carrier solution was pumped by a M930 HPLC pump (Young-Lin Scientific Co., Anyang, Korea). The elution of particles was monitored by a UV-106 UV/VIS detector (Linear Instruments, Reno, USA) operating at the wavelength of 254 nm. The SdFFF system was controlled and the data were collected using a computer and the software programmed in this laboratory. The data processing was performed by the software obtained from Postnova USA (Salt Lake City, Utah, USA). The carrier liquid was water containing 0.1% (w/v) FL-70 (Fisher Scientific, Fair Lawn, NJ, USA) and 0.02% (w/v) sodium azide (NaN_3) as a bactericide. All experiments were performed at room temperature.

Results and Discussion

Dispersing of pollens. Various aqueous media were tested to select a dispersing liquid for pollens. Figure 1 shows UV/VIS absorbance measured at 254 nm continuously for 10 min for the Paper Mulberry pollens dispersed in various aqueous media. All suspensions show similar trends - the absorbance decreases with time at the beginning, due to settling of the pollens, and after about 4 min reaches a steady state.

Figure 2 shows UV/VIS absorbance measured by the same way for the Paper Mulberry pollen dispersed in water containing 0.1% FL-70 after sonication. The sonication time was 0, 1, 3, and 5 min, respectively. All suspensions show similar trends in absorbance as in Figure 1 with no significant differences.

Figure 3 shows UV/VIS absorbance measured again by the same way for Paper Mulberry pollen dispersed in water

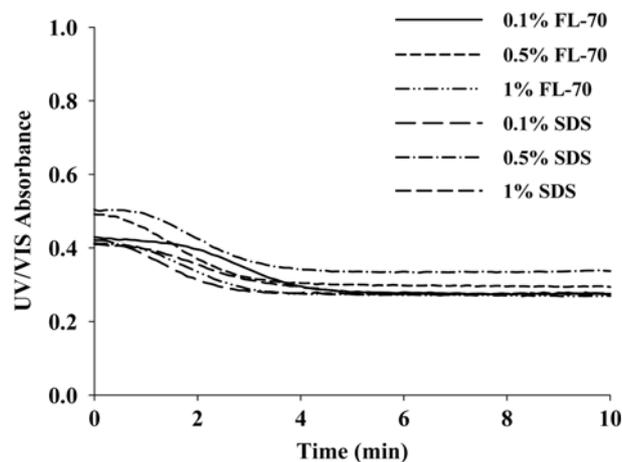


Figure 1. UV/VIS absorbance measured continuously at 254 nm for Paper Mulberry pollen dispersed in various aqueous media.

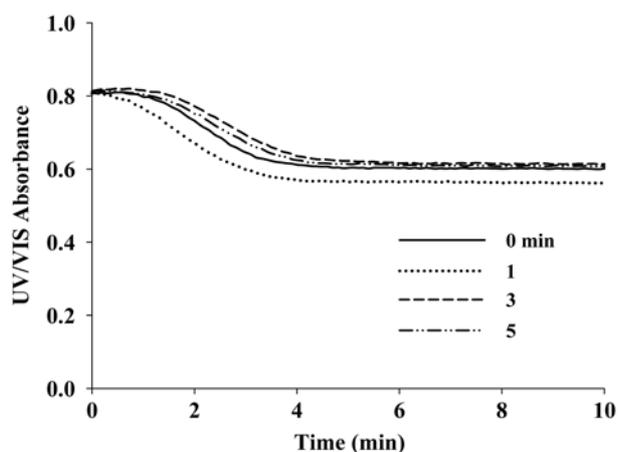


Figure 2. UV/VIS absorbance measured continuously at 254 nm for Paper Mulberry pollen dispersed in water containing 0.1% FL-70 after sonication. The sonication time was 0, 1, 3, and 5 min, respectively.

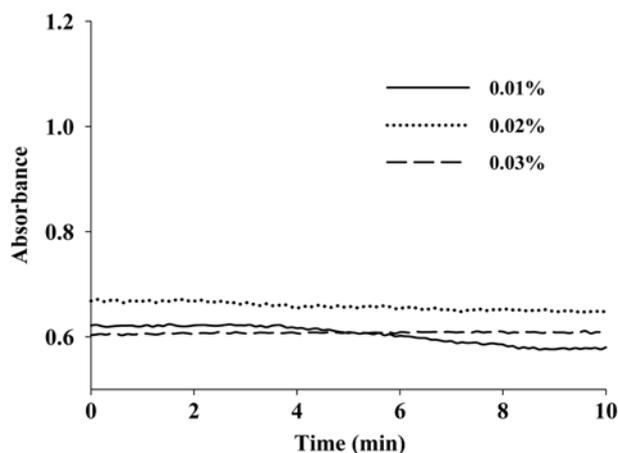


Figure 3. UV/VIS absorbance measured continuously at 254 nm for Paper Mulberry pollen dispersed in water containing 0.1% FL-70 and various concentrations of sodium azide (NaN_3).

containing 0.1% FL-70 and various concentrations of sodium azide (NaN_3), a bactericide. Again, no significant differences were found. Based on the data shown in Figure 1-3, water containing 0.1% FL-70 and 0.02% sodium azide was chosen as dispersing medium (and also as the carrier in GrFFF) for pollens and 3 min as the sonication time in this study.

Optical microscopy analysis of pollens. Figure 4 shows optical microscopy (OM) pictures of the Paper Mulberry and the Bermuda grass pollens dispersed in water containing 0.1% FL-70 and 0.02% sodium azide. Both pollens are in almost spherical shapes. The size distributions of the pollens determined by OM are shown in Figure 5. It can be seen that both pollens have broad size distributions with the average sizes of 13.8 and 28.6 μm for the Paper Mulberry and the Bermuda grass pollens, respectively, which are in good agreements with the nominal values.

GrFFF calibration. Figure 6 shows GrFFF fractograms of four polystyrene latex standards (having nominal diameters of 4.5, 14.9, 21.4, and 45.6 μm) obtained at the flow rate of

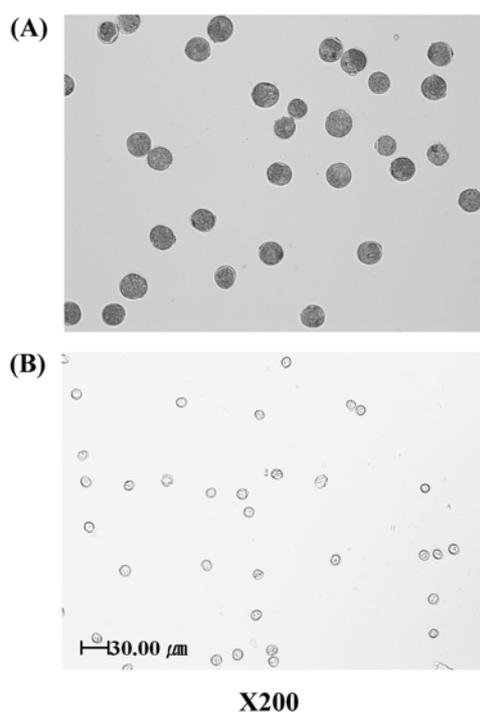


Figure 4. Optical Micrographs (magnification of 200) of Bermuda grass (A) and Paper Mulberry (B) pollens dispersed in water with 0.1% FL-70.

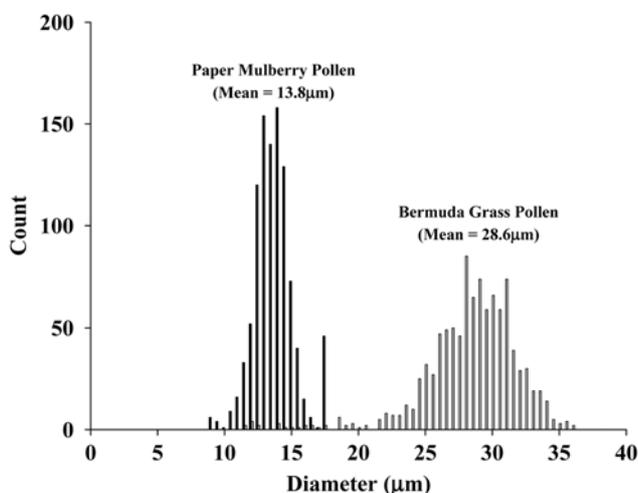


Figure 5. Size distributions determined by OM for Paper Mulberry and the Bermuda Grass pollens.

2 mL/min. Their retention data are summarized in Table 1. As shown in Table 1, the values of γ is different for particles of different sizes. This will cause the calibration plot deviate from linearity. The value of γ increases with size, probably due to increased hydrodynamic lift forces for larger particles.^{26,29,30} Figure 7 shows the calibration plot (plot of $\log t_r$ vs. $\log d$) obtained by using the retention data shown in Table 1. As expected, the data deviate somewhat from linearity, and, when fitted by the first-order least square method, the correlation coefficient was 0.9388. The calibration curve shown in Figure 7 was used for size determination of pollens in this study.

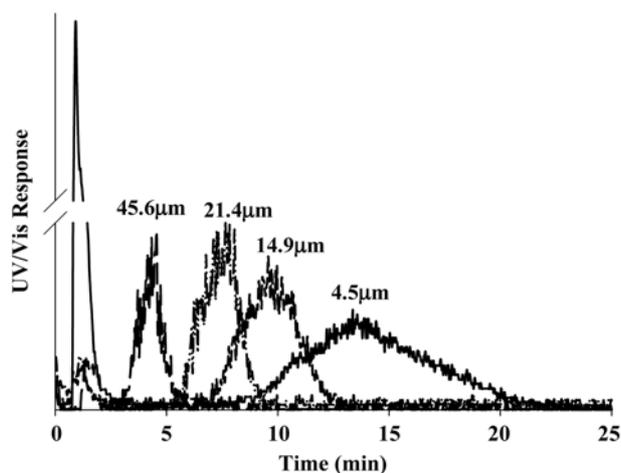


Figure 6. GrFFF fractograms of polystyrene latex standards obtained at flow rate of 2 mL/min.

Table 1. Retention data and the steric correction factor (γ) determined for polystyrene latex standards shown in Figure 6

Nominal diameter (μm)	t_r (min)	R	γ
4.5	15.0	0.096	1.81
14.9	9.60	0.149	0.85
21.4	7.10	0.202	0.80
45.6	4.30	0.334	0.62

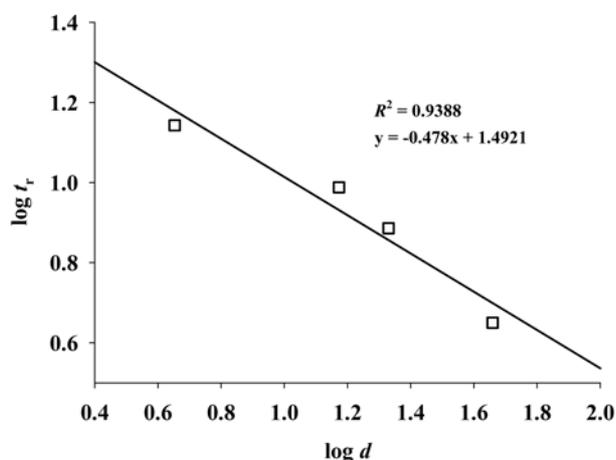


Figure 7. GrFFF calibration curve obtained from retention data of polystyrene latex standards shown in Figure 6.

GrFFF analysis of pollens. Figure 8 shows GrFFF fractograms of the Paper Mulberry and the Bermuda grass pollens obtained at the flow rate of 2 mL/min. Figure 9 shows the number-based size distributions of the pollens obtained by GrFFF along with those obtained by optical microscopy (OM). For the Paper Mulberry pollen, the mean size obtained by GrFFF is 12.7 μm , and agrees rather well with the OM data (13.8 μm). The relative error is 8.0%. For the Bermuda Grass pollen, the mean size obtained by GrFFF is 32.6 μm , and the relative error is 12.3%. These differences between the results from GrFFF and OM may be attributed to that fact that (1) the calibration curve is not linear, and (2)

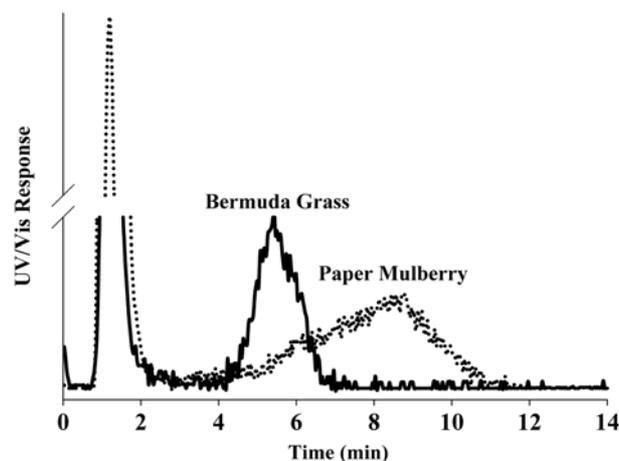


Figure 8. GrFFF fractograms of pollens obtained at the same experimental conditions as in Figure 6.

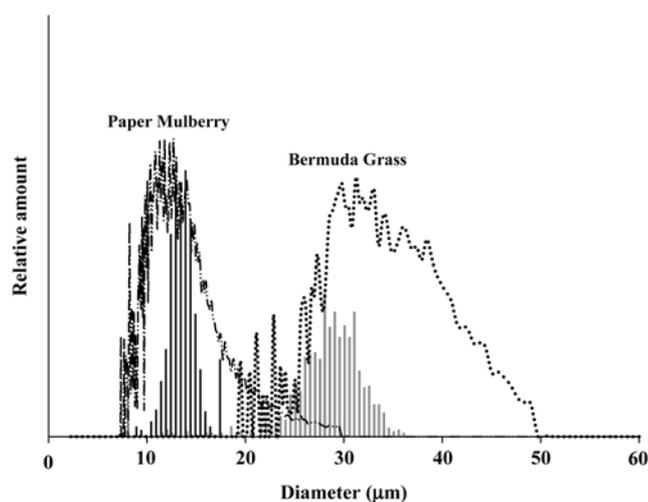


Figure 9. Number-based size distributions of pollens obtained by GrFFF (line) and optical microscopy (bars).

the calibration standards were polystyrene latex beads which are not same as the pollens in chemical composition as well as in physical properties. As shown in Figure 9, the size distributions of both pollens obtained by GrFFF are broader than those from OM, due to band broadening in GrFFF channel during elution.

SdFFF analysis of pollens. One of limitations of GrFFF is that the field strength is not tunable (fixed at 1 gravity). Sedimentation FFF (SdFFF) is another member of FFF. Although the instrumentation and operation of SdFFF are more complicated than those of GrFFF, the separation mechanism of SdFFF is the same as that of GrFFF.⁵ The main difference between two techniques is that, in SdFFF, the field strength can be tuned by adjusting the channel rotation speed.

SdFFF fractograms of the pollens are shown in Figure 10. The channel rotation rate was 400 rpm, which is equivalent to 27 gravity, and the flow rate was 3 mL/min. Figure 11 shows the number-based size distributions of the pollens obtained by SdFFF along with those obtained by optical

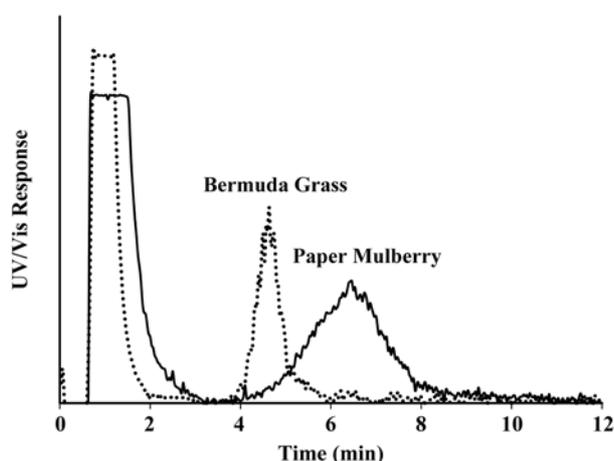


Figure 10. SdFFF fractograms of pollens obtained at the channel rotation speed of 400 rpm (27 gravity) and flow rate of 3 mL/min.

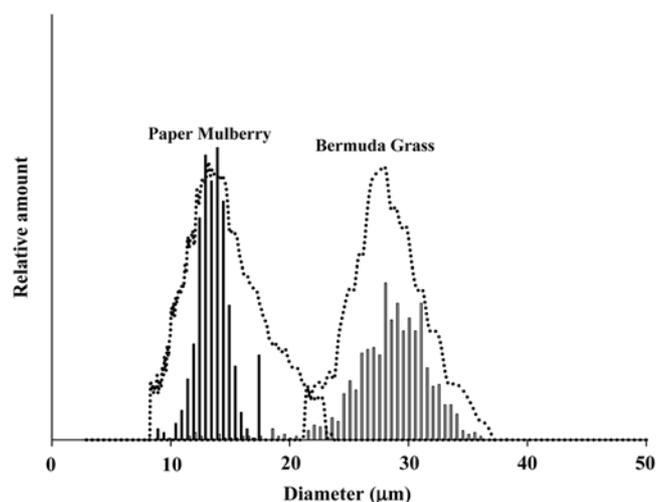


Figure 11. Number-based size distributions of pollens obtained by SdFFF (dotted lines), and by optical microscopy (bars).

microscopy (OM). It can be seen that the band broadening is lower in SdFFF than in GrFFF, especially for the Bermuda Grass pollen, when compared with the data shown in Figure 9.

The mean sizes determined by SdFFF are listed in Table 2, where the mean sizes from GrFFF and OM are also listed. The mean sizes determined by SdFFF are 12.4 (relative error = 10.1%) and 27.1 μm (relative error = 5.2%) for the Paper Mulberry and the Bermuda Grass pollen, respectively. SdFFF yields more accurate sizes for micron-sized pollens in terms of the relative error, probably due to lower band broadening. However the mean sizes from GrFFF and SdFFF are not significantly different from each other.

Conclusion

GrFFF and SdFFF provide size-based separation of micron-sized Paper Mulberry and Bermuda Grass pollens. Both GrFFF and SdFFF yielded the mean sizes that are in reasonable agreements with the results from optical microscopy

Table 2. Mean diameters measured for pollens by GrFFF, OM, and SdFFF

Pollen	Nominal diameter range (μm)	Mean diameter (μm) measured by		
		GrFFF	SdFFF	OM
Paper Mulberry	11~17	12.7	12.4	13.8
Bermuda grass	21~29	32.6	27.1	28.6

(OM), despite that the calibration standards (polystyrene latex beads) were not same as the pollens in chemical composition as well as in physical properties including the density.

Sedimentation FFF (SdFFF) may yield more accurate size distribution of the pollens due to lower band broadening when high field strength (higher than 1 G) is employed. The mean sizes obtained by GrFFF are 12.7 (relative error = 8.0%) and 32.6 μm (relative error = 12.3%), while those by SdFFF are 12.4 (relative error = 10.1%) and 27.1 μm (relative error = 5.2%) for the Paper Mulberry and the Bermuda Grass pollen, respectively. Still, the mean sizes determined by GrFFF were not significantly different from those by SdFFF.

GrFFF is simple in theory, less costly to set up and easier to operate than most other separation techniques including SdFFF. It is also less time-consuming than OM with total analysis time of less than about 12 min. With further optimization of GrFFF by reducing the band broadening, GrFFF could be a useful tool for quick size-analysis of micron-sized pollens and particles of various environmental origins.

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