

The effects of carpet fresheners and other additives on the behaviour of indoor allergen assays

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Summary

Background Chemical agents such as tannic acid and detergents have been shown to introduce non-random bias in allergen measurement.

Objective We investigated how several substances that are commonly found in floor dust (carpet fresheners, powdered pesticides, and table salt) affected immunoassays of purified standard allergens.

Methods Three sets of experiments were conducted to: (1) screen for interference with allergen enzyme-linked immunosorbent assay (ELISA); (2) test for concentration-response; and (3) assess the site-of-action of a given dust additive (i.e. the effect on allergen binding to primary or secondary antibody). The ELISAs are commercially available two-site monoclonal antibody assays for Der p 1, Der f 1, and Fel d 1, and a monoclonal/polyclonal assay for Bla g 1. Outcomes are reported in terms of reaction rate (colour change per unit time), which is directly proportional to the amount of bound allergen.

Results In the initial screening experiments, carpet fresheners tended to decrease Der p 1 assay reaction rates, increase Der f 1 assay rates, and produce little change in Fel d 1 assay rates. Three carpet fresheners decreased Der p 1 assay rate responses in a concentration-dependent manner. Two carpet fresheners noticeably increased Der f 1 assay reaction rates in both the screening and the concentration-response tests. Powdered pesticides increased reaction rates in the Bla g 1 assays and increased the slope of the dilution curve compared with that of the purified allergen. Salt decreased the reaction rates of Bla g 1 assays at allergen concentrations greater than 0.01 U/mL. For each of the four allergens, the largest effects of dust additives occurred when secondary antibody binding was altered.

Conclusions Some common household dust components can introduce systematic error into immunoassays for arthropod allergens.

Keywords: ELISA, Der f 1, Der p 1, Fel d 1, Bla g 1, carpet freshener, allergens

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Introduction

Samples of settled house dust are commonly used for the measurement of allergen levels by enzyme-linked immunosorbent assay (ELISA). Resultant values are then used as a surrogate measure for inhalational allergen exposure [1–4].

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Household floor dust can be a complex mixture of soil, lint, sand, bacteria, fungal spores, human and pet hair, skin scales and secretions, small insects and arachnids (along with their fecal pellets), or other organic material, and may contain relatively large amounts of chemicals added purposely or accidentally. Previous studies have indicated that chemical treatment of carpet to reduce dust mite allergen as well as the presence of household detergents commonly recovered from house dust can affect the performance of ELISAs [5,6]. Effects of other substances that residents add to floor dust

either advertently or inadvertently on the performance of ELISAs are unknown.

Substances such as carpet fresheners, powdered pesticides, and table salt tend to remain in settled floor dust for several reasons. Although manufacturers recommend vacuuming after application of carpet fresheners, some residue is likely to remain. Manufacturers also recommend applying (and not removing) some powdered pesticides (containing boric acid) to locations where cockroaches usually crawl (e.g. kitchen floors). Table salt that has been inadvertently spilled is also commonly found in kitchen dust. Since many epidemiologic studies use floor dust for the measurement of allergens, the effects of these substances on the ELISAs warrant examination. We investigated the effects of carpet fresheners, powdered pesticides, and table salt on assay of the purified standard allergens Der p 1, Der f 1, Fel d 1, and Bla g 1.

Materials and methods

Baking Soda (i.e. sodium bicarbonate), eight different carpet fresheners, table salt (without iodine), and a brand of powdered pesticide were purchased from area stores; boric acid was purchased from Sigma Chemical Company (St Louis, MO, USA). The principal ingredient in carpet fresheners was either baking soda, sodium sulphate, or a mixture of both with trace amounts of different fragrances. The pH of the carpet fresheners ranged from 7.3 to 8.3.

Three sets of experiments were conducted to: (1) identify interference with the ELISA with a single concentration of additive; (2) evaluate concentration-related changes in assay standard curves when contaminants were added; and (3) determine the assay steps that were affected by the additives. Carpet fresheners and baking soda were studied in this manner to evaluate interference with the dust mite (Der p 1 and Der f 1) and cat (Fel d 1) allergen assays, whereas salt, boric acid and powdered pesticide were evaluated only for interference with the cockroach allergen (Bla g 1) assay.

To assess the influence of a carpet additive on actual dust samples, nine house dust samples were obtained from homes with known detectable levels of Der p 1 in bedroom floor dust. The occupants of these homes reported no use of carpet freshener or tannic acid, which is known to interfere with the ELISA [5]. Dust samples from 2 m² of the bedroom floors were collected with a canister vacuum cleaner and cellulose thimble filters during a 5-min sampling period. Each dust sample was sieved through 425- μ m mesh, and the fine dust was stored at -20 °C. Upon rethawing for assay, dust was extracted in borate-buffered saline (BBS, pH = 8.5), centrifuged, and the supernatant was serially diluted for allergen analysis by ELISA [7].

The ELISAs are two-site monoclonal antibody assays for Der p 1, Der f 1, and Fel d 1, and a monoclonal/polyclonal

Bla g 1 assay and were performed as described by the supplier (Indoor Biotechnologies, Clywd, UK) [7–9]. However, rather than using the endpoint method as described, the microtitre plates were immediately placed in a V_{max} microplate reader (Molecular Devices Corp., Menlo Park, CA) and absorbance at 405 nm was recorded every 15 s for a total of 5 min. The plate reader agitated the microtitre plate for 5 s between each measurement in order to ensure thorough mixing of the substrate. The results for each well were expressed as the 'rate response' [maximum rate of reaction in milli-optical density units per minute (mOD)]. Absorbance is not always uniform across the wells of a microtitre plate, due to the manufacturing process. The kinetic assay is more precise than an endpoint assay because it adjusts for well-to-well variability and baseline absorbance.

In order to assess the variability due to extraction of house dust samples, 10 samples were divided into two portions, portions were extracted separately, and were assayed for Der f 1. The relative difference between Der f 1 concentrations of the first and second extract ranged from -15% to +42% (one pair of extracts was below limit of detection [LOD]).

Preliminary experiments

Preliminary experiments were conducted to assess effects of carpet fresheners on dust mite and cat allergen assays. A 25-mg aliquot of a carpet freshener or other additive was added to a solution of known standard allergen (25 ng of Der p 1 or Der f 1/mL of 1% bovine serum albumin in phosphate-buffered saline with Tween 20 (BSA-PBST), or 2.5 mU of Fel d 1/mL of 1% BSA-PBST). For each allergen ELISA, seven replicate microtitre plate wells of each additive-allergen solution were assayed. On each microtitre plate, seven wells of purified standard served as a positive control.

The median rate response of each standard with additive was compared with that of the purified standard using the Wilcoxon rank sum test, which is a more robust method than the *t*-test for comparing differences between samples with and without additives. The Bonferroni correction adjusted for multiple comparisons (i.e. *P*-values less than 0.05/9 = 0.0056 were considered statistically significant).

Concentration-response experiments

Concentration-response experiments were performed with eight carpet fresheners for each of three allergens (Der p 1, Der f 1, and Fel d 1). Similar experiments were conducted using boric acid, a commercial pesticide containing primarily boric acid, and table salt to evaluate effects on the Bla g 1 assay.

For each allergen, a stock solution was prepared (Der p 1 and Der f 1–80 ng/mL, Fel d 1–10 mU/mL, and

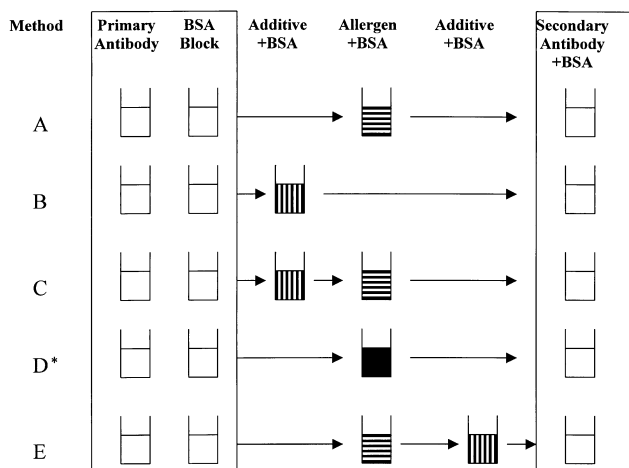


Fig. 1. Site-of-action protocols: modification of the allergen addition step in the standard enzyme-linked immunosorbent assay protocol. *Allergen and additive added together.

Bla g 1–0.08 U/mL). For each additive, 0.3 mL aliquots of these stock solutions were added to each of four vials. In the first three vials, quantities of a given additive were added to obtain concentrations of 25, 12.5, and 6.25 mg of additive/mL. The fourth vial was a positive control that contained allergen without any additive. Seven twofold serial dilutions were made from each of these vials. The solutions were assayed with ELISA, and subsequently new stock solutions were prepared, and the experiments were replicated.

The concentration-response data were analysed using a four-parameter logistic model:

$$f(x, \beta) = \beta_1 + (\beta_2 - \beta_1 / 1 + \exp[\beta_4(\log(x) - \beta_3)])$$

where X is the concentration of the standard, β_1 and β_2 are the rate responses at infinite and zero concentration, respectively; β_3 is the inflection point of the curve; and β_4 is a slope parameter [10]. A feature of immunoassay data is non-constant response variance that increases with response level [11,12]. This was taken into account in the analyses by modelling the variance as a function of $f(x, \beta)$, assuming a power of the mean model [13]. The standard curves with and without additives were fitted using the method of generalized least squares with the pseudolikelihood estimation procedure to estimate the variance parameter [13].

Separate curves were estimated for the standard allergen dilution series with (1) no additive; (2) a 'high' concentration of additive (25 mg/mL); (3) a 'medium' concentration of additive (12.5 mg/mL); and (4) a 'low' concentration of additive (6.25 mg/mL). Differences between the four curves were identified by choosing a rate response (200 mOD units/min) and locating its corresponding allergen concentration on each of the four estimated curves via inverse regression [13].

Site-of-action experiments

Site-of-action experiments were conducted to determine the steps in each assay where effects were occurring. Some additives that did not appear to affect the assays, as determined from the preliminary and concentration-response experiments, were also evaluated with this method to serve as negative controls. A site-of-action experiment was not conducted with pure boric acid, since the powdered pesticide contained >99% boric acid and both substances exhibited similar effects for the the concentration-response experiments.

A solution of additive was prepared at a concentration of 25 mg/mL of buffer. A second solution contained only standard allergen (Der p 1 and Der f 1–25 ng/mL, Fel d 1–2.5 mU/mL, and Bla g 1–0.02 U/mL). A third solution contained a mixture of additive and standard allergen (e.g. 25 mg of additive + 25 ng of Fel d 1 in 1 mL of BSA-PBST). These solutions were assayed using five different methods, with four replicates each (Fig. 1).

Method A (positive control)

Purified allergen without additive was assayed by the standard ELISA procedure described previously. This was a positive control to which all rate responses were compared.

Method B (negative control)

The solution with only additive was assayed. This was a negative control for non-specific binding of secondary antibody to the microtitre plate and/or contamination of the additive by the allergens under investigation.

Method C (additive first)

The solution with only additive was added and the plate was washed, then the solution with only allergen was added and assayed.

Method D (mix)

The allergen and additive were mixed and added to the microplate as a single solution. This method is identical to the method of allergen/additive application used in the preliminary experiment.

Method E (allergen first)

The solution with only allergen was added and the plate was washed, then the solution with only additive was added and assayed.

A Wilcoxon signed rank test was used to test, for all

Table 1. Effect of carpet fresheners on dust mite and cat allergen enzyme-linked immunosorbent assays: mean rate response of seven replicates at 25 ng/mL.

Additive	Der p 1		Der f 1		Fel d 1	
	Mean (mOD units/min)*	SD	Mean (mOD units/min)*	SD	Mean (mOD units/min)*	SD
<i>Reference</i>						
No additive	165	3.4	164	3.2	102	2.0
<i>Sodium bicarbonate</i>						
X1 (pH 8.3)	105†	1.5	197†	3.8	100	1.1
X2 (pH 8.3)	107†	2.1	199†	4.3	99	3.1
Mean response	106		198		99	
<i>Sodium bicarbonate and sodium sulphate</i>						
Y3 (pH 8.1)	121†	2.5	176†	4.9	95†	2.0
Y4 (pH 8.0)	124†	2.5	177†	3.6	99	3.2
Y5 (pH 8.0)	126†	2.6	181†	6.0	99	2.4
Y6 (pH 8.0)	131†	3.5	179†	4.1	101	2.3
Mean response	125		178		98	
<i>Sodium sulphate</i>						
Z7 (pH 7.3)	155†	3.5	158	2.0	104	1.9
Z8 (pH 7.4)	157	6.5	160	3.8	103	3.3
Z9 (pH 7.4)	159	4.6	169	3.9	105	2.4
Mean response	157		162		104	

* mOD units/min = rate response obtained from the plate reader. † Significantly different from reference response (Wilcoxon rank sum test $P < 0.0056$).

additives, which methods resulted in data that were significantly different from those of the positive control method, assuming the additives were a random sample of carpet fresheners. A Bonferroni correction adjusted for the comparisons of the three methods to the positive control [i.e. P -values less than 0.017 ($= 0.05/3$) were considered statistically significant]. Results were listed as percentage change from the mean response of the positive control method.

House dust experiments

For each of nine floor dust samples, 25 mg of house dust was combined with 25 mg of baking soda; 50 mg of house dust alone served as a matched control. A 1 mL aliquot of BBS was added to each vial and extracted. The vial with 50 mg of house dust would have twice as much allergen per mL as the vial with 25 mg of house dust plus 25 mg of baking soda, so it was diluted twofold after the extraction procedure to produce the same concentration of allergen per mL as the solution with 25 mg of baking soda. Matched solutions were assayed at full strength, and at dilutions of 1:2, 1:4 and 1:8. Further dilutions were made when samples were above the assay's upper limit of detection. A Wilcoxon signed rank test was used for comparing rate responses of paired samples with and without baking soda.

Results

Carpet fresheners and baking soda

Preliminary experiments with carpet fresheners and Der p 1, Der f 1, and Fel d 1 showed that overall, interfering substances tended to decrease the Der p 1 rate responses, increase the Der f 1 rates, and produce little change in the Fel d 1 rates (Table 1). Further analysis confirmed that X1 (baking soda), X2, and Y5 decreased the Der p 1 rate responses in a concentration-dependent manner (Fig. 2). In the case of Der p 1, when a standard allergen solution contained 40 ng/mL with no baking soda, allergen solutions with the equivalent of 50% baking soda (w/v) resulted in a lower estimate of 20 ng/mL. With only 25% baking soda (w/v), this effect diminished to 25 ng/mL, and with 12.5% baking soda (w/v), the effect diminished to an estimate of 30 ng/mL (which was still lower than the concentration of purified allergen).

Baking soda and all of the carpet fresheners containing baking soda increased Der f 1 rate responses in the preliminary experiments (Table 1), and two of the carpet fresheners also increased rate responses in the concentration-response experiments (Fig. 3). Y3 significantly decreased Fel d 1 rate responses in the preliminary experiments, but the decrease was not as pronounced with the concentration-response

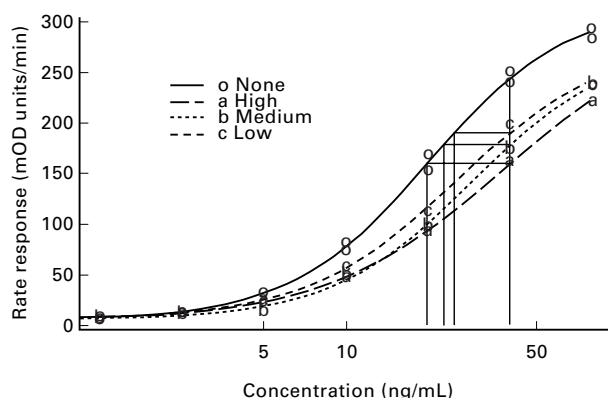


Fig. 2. Concentration-response curve for Der p 1 and concentrations of baking soda (additive X1) ranging from 0 mg/mL to 25 mg/mL. Vertical lines intersecting the x-axis indicate the 40 ng/mL Der p 1 concentration relative to the standard curve containing no baking soda.

experiments. The carpet freshener, Y5, resulted in a slight change of Fel d 1 rate responses in both the preliminary and concentration-response experiments.

Results from site-of-action experiments are shown in Fig. 4. Compared with Method A (positive control), Methods D (Mix) and E (Allergen 1st) decreased Der p 1 responses, and Method C (Additive 1st) increased Der p 1 responses. Most of the carpet fresheners increased Der f 1 responses via Method D. However, the differences were not significant. Methods D and E significantly decreased Fel d 1 responses.

Pesticides and salt

Bla g 1 standard curves were altered by the addition of the powdered pesticide as well as pure boric acid (Fig. 5). At

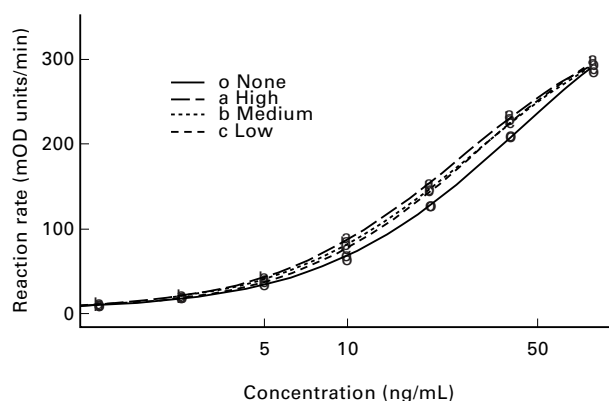


Fig. 3. Concentration-response curve for Der f 1 and concentrations of carpet freshener (additive Y3) ranging from 0 to 25 mg/mL.

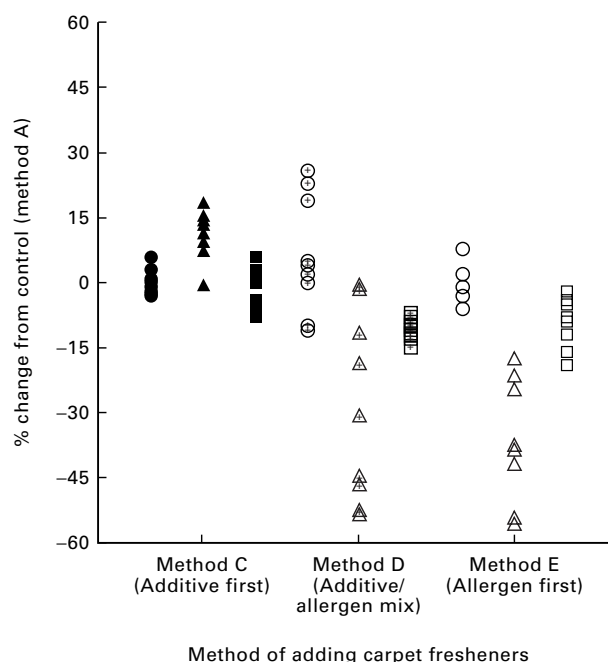


Fig. 4. Site-of-action rate responses: percent change from positive control (Method A). Circles (●) indicate Der f 1 responses, triangles (▲) indicate Der p 1 responses, and squares (■) indicate Fel d 1 responses. Each column contains a point representing the average percentage change in rate response relative to the positive control for each of the eight carpet fresheners.

higher concentrations, the slopes increased and yielded higher responses than did the standard curve. At Bla g 1 concentrations greater than 0.01 U/mL, salt decreased rate responses. Salt decreased Bla g 1 responses by Methods C, D and E of the site-of-action experiments by -4%, -30%, and -20%, respectively, while powdered pesticide increased

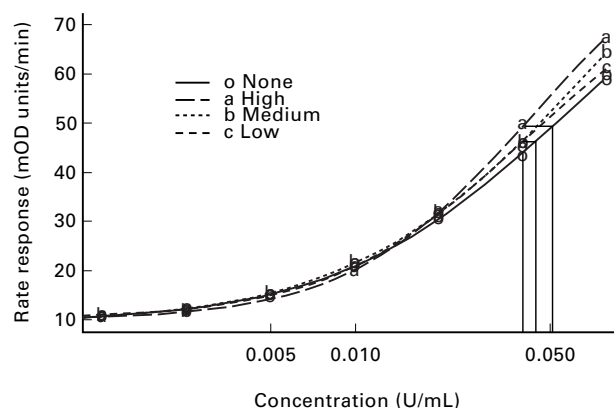


Fig. 5. Concentration-response curve for Bla g 1 and concentrations of powdered pesticide ranging from 0 to 25 mg/mL. Vertical lines intersecting the x-axis indicate the 0.04 U/mL Bla g 1 concentration relative to the standard curve containing no powdered pesticide.

Table 2. Der p 1 content in house dust extracted with and without baking soda.

Dust sample	Dust without baking soda (control) ($\mu\text{g/g}$)	Dust with baking soda	
		Adjusted for weight of baking soda ($\mu\text{g/g}$)	Not adjusted for weight of baking soda ($\mu\text{g/g}$)
1	0.89	0.80	0.40
2	1.40	2.38	1.19
3	1.45	1.09	0.55
4	1.96	1.50	0.75
5	2.60	1.33	0.67
6	6.42	6.32	3.16
7	7.03	5.86	2.93
8	13.1	11.7	5.85
9	30.0	27.9	14.0
Mean	7.21	6.54*	3.27*

* Der p 1 in dust samples with baking soda was significantly different from Der p 1 in control samples (Wilcoxon signed rank test $P = 0.005$).

responses via Methods C, D and E by +4%, +17%, and +1%, respectively.

Actual house dust samples

Because the greatest change in rate responses consistently occurred when baking soda was included in the Der p 1 assays, the implications of these results were examined using actual samples of house dust. Baking soda decreased the calculated concentrations of Der p 1 in house dust eight times out of nine. A Wilcoxon signed rank test indicated the median concentration of samples with baking soda was significantly lower than that of the matched control samples (Table 2).

Discussion

Immunoassays depend on the binding affinity of highly specific sites on antigens and antibodies and on the absence of non-specific binding to the plate itself. Dust is a complex mixture of substances that might interfere in the assays either by changing the conformation of binding sites, by removing primary antibodies or previously bound allergen, or by promoting non-specific binding. Our studies demonstrate such effects on assays for four common indoor allergens. Our data have important implications both with respect to measurement of allergen and to allergen exposure because changes in the characteristics of allergens may alter human responses as well as assay responses.

Additives generally decreased rate responses in Der p 1 assays, increased rate responses in the Der f 1 assays, were additive-specific for the cockroach assays, and had little

effect on Fel d 1 assays (except for some significant decreases during the site-of-action experiment). In most cases, additives appeared to affect the allergens directly, preventing or enhancing binding to primary or secondary antibodies. Most assay effects were seen when additives were either mixed with the allergen (Method D) or added to the plate following allergen addition (Method E). In both scenarios, additives came into direct contact with binding sites on the allergens. Although non-specific binding of the allergen directly to the plate is another plausible reason for increases in rate responses compared with the positive control (Method A), non-specific secondary antibody binding is unlikely, given that the additive in the negative control (Method B) resulted in no detectable rate response. Both increases and decreases in rate responses could be hypothesized to result from changes in epitope conformation or presentation, and both of these changes could possibly affect human responses to allergen.

As demonstrated by the concentration-response curves, boric acid and powdered pesticide caused overestimation of levels of Bla g 1 up to 17% (using a Bla g 1 concentration of 0.02 U/mL and 25 mg/mL of additive). This effect may cloud the relationship between powdered pesticide use and levels of Bla g 1 as a marker of cockroach presence. The fact that boric acid is used may indicate the presence (or previous presence) of cockroaches. On the other hand, the use of boric acid may result in increases in measured levels directly through effects on the assay. In addition, this effect may indicate that the use of boric acid for cockroach control may enhance the potency of residual cockroach allergen with respect to stimulating human immune responses.

The inhibitory effect of salt in the Bla g 1 assay could also interfere with interpretation of data. Exposure misclassification with respect to Bla g 1 would occur randomly if salt is accidentally spilled in some kitchens. However, systematic bias could occur in regions of the world where salt is added to the roads during the winter. Salt is tracked into homes in such areas, and Bla g 1 levels in dust samples could be underestimated with respect to areas not using salt, or in winter as opposed to summer samples.

Baking soda and carpet fresheners containing baking soda exerted the greatest effect on the dust mite assays, while carpet fresheners in which the primary ingredient was sodium sulphate were generally less potent. Some of the greatest changes in rate response occurred with the most basic solutions. This effect paralleled the amount of baking soda in the products. However, previous research has suggested that pH alone does not significantly change the measurement of allergens by ELISA [5].

Some carpet fresheners changed the slope and inflection point of concentration-response curves. In the Der f 1 experiments, the dilution curves for all three concentrations of Y4 and Y3 were above the standard curve but not necessarily in a concentration-dependent manner. This response could be attributed to a threshold effect.

Changes in Fel d 1 assay rate responses in the presence of carpet fresheners were minimal and inconsistent. However, there may be other dust additives that could affect this ELISA. For example, tannic acid has been shown to affect the binding of Fel d 1 to the same monoclonal antibodies used in our study [5].

Although we clearly demonstrated significant effects on these allergen assays, many of the effects were small, and resulted in only small changes in allergens assayed from actual dust samples (Table 2). Van Strien *et al.* reported a coefficient of variation (CV) for Der p 1 concentrations in duplicate extracts to be 25.3% (CV = 1.7% in transformed) [14]. Also, Wickens *et al.* reported a CV of <15% [15]. Perhaps the most relevant conclusion from this variability data is that replicate dust sample extractions and replicate assays of the same extract result in calculated values that are randomly above or below the original sample, whereas the carpet fresheners tend to produce a systematic increase in Der f 1 concentrations and a systematic decrease in Der p 1 concentrations.

Table 2 also lists values of Der p 1 per gram of dust when the weight of the baking soda was included in the analysis. It should be noted that the purposeful addition of any substance (inert or not) will change the ratio between the amount of allergen and the total amount of dust collected. This is an especially important consideration for intervention studies where allergen levels are measured before and after addition of treatment compounds. We agree with Woodfolk *et al.* [5] that a consideration of contaminants

known to exert effects on the ELISA should be a part on the interpretation of calculated allergen concentrations. However, the concentration of the additive in house dust would require estimation.

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