Background: Sublingual immunotherapy (SLIT) has become established in Europe, and its efficacy is being evaluated in the United States. The doses used for SLIT in Europe today are difficult to evaluate, because each manufacturer expresses the potency of its extracts differently.

Objectives: To compare in vitro European SLIT maintenance solutions against US licensed standardized allergenic extract concentrates and to determine the monthly SLIT doses delivered expressed in bioequivalent allergy units (\([B]\)AU).

Methods: We studied *Dermatophagoides pteronyssinus*, timothy grass pollen, cat (hair) and short ragweed pollen allergen extracts. The SLIT maintenance solutions of 4 leading European manufacturers and standardized concentrate extracts of 3 US manufacturers were analyzed with the following assays: protein content, relative potency (immunoglobulin E [IgE]-binding enzyme-linked immunosorbent assay [ELISA] inhibition) and major allergen content. The relative monthly allergen dose in \((B)AU\) was calculated for each recommended SLIT schedule.

Results: Relative potency was approximately 10 times higher for US concentrate standardized extracts—which are meant to be diluted—than for European SLIT maintenance solutions of *D pteronyssinus* and timothy grass pollen. For cat (hair) and short ragweed pollen, the difference was less. Measurements of relative potency and major allergen content correlated well. In our assays, European mite extracts contain a very low quantity of Der p 2 compared with US mites.

Conclusion: Recommended SLIT doses in Europe vary widely among the manufacturers, but are consistently lower (Eur1) or higher (Eur4) over all four allergens tested. SLIT efficacy probably depends on additional factors apart from the exact dose. SLIT dose finding studies should be done for each product.

INTRODUCTION

Subcutaneous immunotherapy (SCIT) in the United States consists of the administration of aqueous allergen solutions, derived from glycerinated concentrate extracts, standardized according to the Center for Biologics Evaluation and Research (CBER) of the Food and Drug Administration (FDA) guidelines. Dosing recommendations in the United States for SCIT\(^1\) are inferred from efficacy data mostly obtained from trials with European extracts,\(^2-7\) with the exception of the dosing recommendations for cat and short ragweed pollen, for which the dosing is based on trials with US extracts. The only study published on SLIT using a US extract has been carried out with a sublingual short ragweed pollen solution, based on the same glycerinated allergen extract used for the preparation of SCIT.\(^8\)

For immunotherapy in Europe, glycerinated products are administered, just as in the United States, although extracts are standardized according to in-house-references of each manufacturer.\(^9\) In contrast to in the United States, however, for SCIT almost all extracts are adsorbed to a depot. This is not the case for SLIT extracts, and as such European SLIT solutions bear a close resemblance to US concentrate extracts, both containing a lyophilized allergen dissolved in 50% glycerin. Many clinical trials have been conducted with European SLIT solutions,\(^10-12\) and each manufacturer has partly based the dosing of its SLIT products on these results.\(^11,12\) Several of these trials have been included in a recent updated Cochrane meta-analysis on SLIT.\(^13\)

However, a universal potency measure\(^14\) to quantify the allergen quantity administered by each manufacturer’s solution is lacking, and different designs of the many studies obfuscate the drawing of straight dosing conclusions that might apply to all solutions.\(^15,16\) Some international guidelines have given recommendations for SLIT doses in relation to SCIT doses.\(^16\) Lately, clinical trials with grass SLIT tablets
have shown conclusive effective doses for adults\textsuperscript{17,18} and children\textsuperscript{19,20} but how and whether these data can be extrapolated to SLIT solutions is not clear.

In an ideal world, all laboratories would use the same assays and reagents, generating reliable data on the concentration of major allergens in extracts. This would provide the international intellectual community with accurate information on dose efficacy for European SLIT products, and with the many European SLIT trials conducted, making a clear statement on how much US allergen extract should be given daily to attain a probably effective SLIT dose should be possible. However, reality is far from this ideal scenario.

Allergens are complex biological substances to which each individual can react in a different manner. Thus, not one laboratory assay has been able to fully express all aspects of extracts’ potency. As a consequence, the average result of the outcomes of several in vitro tests might give the most complete picture. The CBER/FDA dictates which assays and reagents are suitable to assign (Bioequivalent) allergy units (BAU and AU) to the commercial batches released onto the American market. For this study, various qualitative and quantitative assays were carried out, including the CBER/FDA tests.

The objective of the current study is to estimate the monthly dose given at SLIT maintenance with the products of prominent European manufacturers, as expressed in US dosing units. As such, we decided to analyze European SLIT products from several major European allergen manufacturers, with assays usually employed to determine the potency of US extracts, including those dictated by the CBER/FDA. To establish this direct potency comparison with the US standardized extracts, analyzing laboratories were asked to run the potency tests they normally run for batch release, simultaneously, on all US and European extracts under investigation.

**METHODS**

Standardized sublingual maintenance extracts were purchased from 4 prominent European allergen manufacturers. Commercial standardized extracts including dust mite (Dermatophagoides pteronyssinus), timothy grass pollen (Phleum pratense), cat (hair) (Felis sp), and (short) ragweed pollen (Ambrosia artemisifolia) were obtained from 3 US manufacturers, and reference extracts of these 4 allergens were acquired from CBER/FDA (Table 1). Analysis took place in the laboratories of the Mayo Clinic, the Children’s Mercy Hospital, CBER/FDA, ALK-Abelló Inc., Greer Laboratories, and Hollister-Stier Laboratories. Each laboratory performed only those analyses that were already run routinely. Because the imprecision of the analyses goes beyond the minor lot-to-lot variability of standardized extracts,\textsuperscript{21} only 1 lot of each was tested. Conversely, each assay was performed at least twice; values reported in the tables and figures are mean results.

**Protein content (qualitative and quantitative)**

Two-dimensional gels were used to analyze the quality of the extracts showing the different proteins in 1 of the laboratories. The protein bands were visualized with Silver Stain. The conditions for running these gels were optimized to demonstrate the entire range of protein content in the samples, rather than being optimized for the best separation of specific allergens. To adjust for the protein content, some extracts were first diluted. A 3,000-kDa cutoff dialysis membrane was used to prepare the samples before electrophoresis. For detailed description of the methods, see e-Methods online.

**Relative Potency Testing**

The relative potency was determined for *D pteronyssinus* and timothy grass pollen extracts. Laboratories 1, 2, 3, and 5 used competition enzyme-linked immunosorbent assays (ELISAs) to measure the inhibition of allergen-specific immunoglobulin E (IgE) binding with respect to current FDA references, using a parallel line assay. Laboratory 4 performed ELISA inhibition with a kit from Indoor Biotechnologies (Charlottesville, Virginia). The results were multiplied by 10,000 to yield AU/mL for the *D pteronyssinus* extracts and by 100,000 to give BAU/mL for the timothy grass pollen extracts.

**Major Allergen Content**

For cat and short ragweed pollen extracts, only the amount of major allergen was examined. The major allergen content was determined by a radial immunodiffusion assay in almost all laboratories, with the exception of laboratory 1, which used for cat the ALK-Abelló Fel d 1 ELISA and converted the micrograms per milliliter obtained into Fel d 1 units with the following calculation: 1 µg Fel d 1 = 0.4 Fel d 1 units.

The major allergen content of *D pteronyssinus* and timothy grass pollen extracts were studied in 2 laboratories. Laboratory 1 uses reagents from ALK (ALK-Abelló, Madrid, Spain), and Laboratory 3 determined all microgram major allergen data with the sandwich ELISA kits from Indoor Biotechnologies. For *D pteronyssinus*, standard curves used in the CREATE project (full project name: Development of Certified Reference Materials for Allergenic Products and Validation of Methods for their Quantification) were applied by laboratory 1. Laboratory 3 used reference standards included with the kits from Indoor Biotechnologies (Der p 1 standard for mite, group 1 allergen, Phil p 5 standard for timothy grass extract, and a Universal Allergen Standard for cat and mite group 2 allergen). The concentration of Phil p 5...
Table 1. Manufacturer, Allergen, Trade Name, and Concentration of the Allergen Extracts Analyzed in the Presented Assays

<table>
<thead>
<tr>
<th>Extract</th>
<th>D. pteronyssinus</th>
<th>Timothy</th>
<th>Cat</th>
<th>Ragweed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manufacturer</td>
<td>Trade name, allergen</td>
<td>Potency</td>
<td>Trade name, allergen</td>
<td>Potency</td>
</tr>
<tr>
<td>CBER/FDA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Reference D. pteronyssinus</td>
<td>5,000 AU/mL</td>
<td>Reference timothy</td>
<td>100,000 BAU/mL</td>
</tr>
<tr>
<td>ALK-USA</td>
<td>Diagnostic concentrate D. pteronyssinus</td>
<td>5,000 AU/mL</td>
<td>Diagnostic concentrate timothy</td>
<td>10,000 BAU/mL</td>
</tr>
<tr>
<td>Greer</td>
<td>Diagnostic concentrate D. pteronyssinus</td>
<td>10,000 AU/mL</td>
<td>Diagnostic concentrate timothy</td>
<td>100,000 BAU/mL</td>
</tr>
<tr>
<td>Hollister-Stier</td>
<td>Diagnostic concentrate D. pteronyssinus</td>
<td>10,000 AU/mL</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>ALK-Euro</td>
<td>SLITone D. pteronyssinus</td>
<td>10,000 STU/mL</td>
<td>SLITone timothy</td>
<td>1,000 STU/mL</td>
</tr>
<tr>
<td>Bencard&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Oralvac D. pteronyssinus</td>
<td>320,000 TU/mL</td>
<td>Oralvac, B2-grass&lt;sup&gt;**&lt;/sup&gt;</td>
<td>768,000 TU/mL</td>
</tr>
<tr>
<td>HAL&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Sublivac D. pteronyssinus</td>
<td>10,000 AU/mL</td>
<td>Sublivac grass</td>
<td>10,000 AU/mL</td>
</tr>
<tr>
<td>Stallergènes&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Staloral D. pteronyssinus</td>
<td>300 IR/mL</td>
<td>Staloral timothy</td>
<td>300 IR/mL</td>
</tr>
</tbody>
</table>

<sup>a</sup> Reference extracts of the FDA were E8-Dp for Dermatophagoides pteronyssinus, E8-Ti for timothy, E5 cat hair for cat, and E15-Ras for short ragweed.

<sup>b</sup> B2-grass of Bencard is a mixture of several grasses.

<sup>c</sup> According to product characteristic leaflet these extracts do not contain phenol as preservative.
was calculated with the CREATE-Allergopharma reference by laboratory 1 and the Indoor Biotechnologies reference by laboratory 3.

Pearson correlations between the different assays were calculated \( (R^2) \) for each allergen, using the mean values of the results per test.

**Monthly SLIT Maintenance Dose Expressed in Potency Measurements Used in the United States**

The relative monthly SLIT maintenance dose for each SLIT extract was calculated based on 2 variables: the relative potency found for each extract in our study (AU, BAU, Fel d 1 U, and Amb a 1 U/mL for *D. pteronyssinus*, timothy grass pollen, cat, and short ragweed pollen extracts, respectively) and the dosing recommendations of each manufacturer concerning frequency and volume of extract given in each dose. We used the following calculation:

\[
\text{Relative potency (BAU/mL)} \times \text{Volume of each dose (mL)} \times \text{Number of doses per month} = \text{Monthly dose (BAU)}
\]

**RESULTS**

**Protein Analyses**

On sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels with silver staining, European and US extracts show high similarity for timothy grass pollen and short ragweed pollen (Fig 1). The SDS-PAGE analysis for

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*Figure 1. SDS-PAGE of European SLIT maintenance extracts and US concentrates of A, *Dermatophagoides pteronyssinus*; B, Timothy grass pollen; C, cat; and D, ragweed pollen.*
US extracts of *D pteronyssinus* and cat revealed a greater number of individual peptide bands, with the exception of the FDA cat hair extract, which showed fewer.

The protein content of US standardized extract concentrates is generally higher than that of European SLIT maintenance extracts. Strong variation exists among the different methods. However, the relative values for protein content (data not shown) when determined by the same method correlate quite well with the other potency data (see below). The Lowry assay results were excluded, because they appeared to be affected by the phenol content present in most of the samples.

**Relative Potency Testing and Content of Major Allergens**

In Figure 2, the relative potency results of the extracts of the 4 allergens are shown. The recommended monthly subcutaneous immunotherapy doses for the US standardized extracts are depicted as small bars to the right of the concentrates: for house dust mite, 500 to 2,000 AU; timothy grass pollen, 1,000 to 4,000 BAU; cat, 1,000 to 4,000 BAU (≈ 1.9–7.6 Fel d 1 U); and short ragweed pollen, 1,000 to 4,000 AU (6–12 Amb a 1 U). The relative potencies of the US *D pteronyssinus* and timothy grass pollen extracts were more than 10 times higher than those of the corresponding SLIT maintenance extracts from the 4 European manufacturers tested.
Moreover, the slopes of the regression lines generated by the European *D pteronyssinus* extracts were not parallel to those of the US *D pteronyssinus* extracts, suggesting that they were qualitatively different as well.

The radial immunodiffusion results for cat and short ragweed pollen extract demonstrated higher potency for some European extracts in comparison with others. The more potent European extracts of these 2 allergens were comparable in potency to the US standardized extract concentrates. For *D pteronyssinus* and cat extracts, approximately 50% variability was found between the US extracts.

Figure 3 shows the Der p 1 and Der p 2 content of European and US extracts, as analyzed by the 3 different laboratories. The mean content of the major allergens Der p 1, Der p 2, Phl p 5, and Fel d 1 can be found in Table 2.

**Relative Monthly SLIT Maintenance Dose (Expressed as BAU)**

Table 3 describes the total quantity of allergen given at SLIT maintenance during 1 month of treatment, based on the outcomes of the relative potency tests and the European manufacturers’ package insert recommendations on dosing.

**Correlations between Potency Measurements**

How the different methods of measuring allergen extract potency correlate with one another is shown in Table 4. In the upper part of the table, the results of protein content are correlated with the relative potency measurements and the content of major allergens. In the lower part of the table, correlations between the latter 2 are shown.
DISCUSSION
To our knowledge, this is the first report of US allergen extracts directly compared with European immunotherapy solutions in laboratory testing, carried out with the same methods in the same laboratories. For the European extracts, we could not use SCIT solutions, because these contain modified depot extracts, so we chose the SLIT maintenance solutions, because these contain natural allergens dissolved in glycerin, just as the US commercial extracts. This allowed us to compare their quality and potency and make a rough estimate of how much allergen is given at SLIT maintenance by the different European manufacturers in terms of (bioequivalent) allergy units. In one of our previous studies, US and European diagnostic extracts were compared, showing in general that US diagnostic extracts of house dust mite, cat, and Bermuda grass pollen are at least twice as potent as European extracts when tested in vitro. Another group of investigators studied laboratory characteristics and potency of European timothy pollen extracts, demonstrating that potency varied considerably among products from different allergen manufacturers. Mösges et al. compared SLIT maintenance solutions of 2 major European allergen manufacturers in quantitative skin prick testing, again showing significant potency differences.

Relative Dosing of SLIT by the Four European Manufacturers Studied
The SLIT maintenance extracts are less potent than the US concentrates, because the latter are meant to be diluted before administration. When extracts are compared, what really is of
interest is the dose that is actually delivered to the patient. As such, in Table 3 we compare the maintenance monthly SCIT dose recommended in the latest update of the Practice Parameters on Immunotherapy\(^1\) with the maintenance monthly SLIT dose that is given with the different products, following the manufacturers’ recommendations. A major difference can be seen between the maintenance SLIT doses given by the 4 European manufacturers studied here. This difference is highly consistent over the 4 allergens we analyzed: *D. pteronyssinus*, timothy grass pollen, cat hair, and short ragweed pollen, with Europe 1 giving low dose SLIT and Europe 4 high dose SLIT.

Sublingual immunotherapy dosing has been addressed in several previous publications, and in the past it has been 1 of the points raised against this form of immunotherapy, because no clear dosing guidelines existed.\(^10,25\) However, that a relatively high dose is needed for SLIT to be effective is generally accepted. In the first Allergic Rhinitis and its impact on Asthma (ARIA) guidelines, dosing of 50 to 100 times the SCIT dose was recommended for SLIT,\(^16\) although in the latest ARIA update,\(^26\) no absolute numbers are given anymore. The World Allergy Organization consensus paper on sublingual immunotherapy mentions the probable effective monthly dose for timothy grass pollen SLIT to be approxi-
mately 600 μg of the major allergen Phl p 5.27 However, the optimal dose has not yet been firmly established for all extracts. Since 2006, convincing data have been generated for some pollen extracts that leave no doubt that the greatest improvement is seen only in the high-dose groups. Almost all of these latter studies are done with tablets.17–20,28,29 One of the few exceptions was the dose-finding SLIT study in children of Valovirta et al,30 which used a liquid solution of tree pollen extract. Recently, a US sublingual standardized glycerinated short ragweed pollen allergenic extract containing 4.8, or 48 μg of Amb a 18 (approximately 10 and 100 times, respectively, of the recommended SCIT monthly dose) administered daily in rhinitis patients showed only in the high-dose group both a 15% reduction in nasal symptom score and a statistically significant reduction of 51% in the medication score during the peak pollen season. The final analysis of our study indicates that high dosing in SLIT is not universally applied in Europe at this moment.

Quality of the Extracts
The data derived from the analysis of the quality of the extracts is also of importance for subcutaneous immunotherapy, as has been previously discussed by Esch.31 To give only 1 example, for the US house dust mite (HDM), dosing recommendations are based on studies with European extracts, as discussed in the introduction. Extrapolating doses for HDM like this is problematic, because our study demonstrates notable qualitative differences.

We analyzed US concentrate and SLIT maintenance extracts with various specific and nonspecific laboratory assays. For HDM, European extracts seem to be different in composition, as indicated by the SDS-PAGE gels (Fig 1) and by the nonparallelism in the relative potency tests (ELISAs). This is confirmed by the specific tests that determine the content of the 2 major allergens, Der p 1 and Der p 2, with the latter lacking almost completely in most European extracts. For cat extracts, differences exist between solutions that are extracted from cat hair or cat epithelium. For timothy grass and short ragweed pollen, the European and US extracts are more alike, according to the gels and the parallelism in the ELISA lines. Also, for these 2 extracts, the different analyzing laboratories gave potency test results within a smaller range. Even though 1 of the European solutions was extracted from the pollen of 12 grasses and not just from timothy grass pollen, it showed a remarkably similar SDS-PAGE protein band pattern to the other extracts.

Protein Analysis
The absolute protein content of extracts is a poor measurement of potency. Moreover, we came across some specific problems: phenol, used as a preservative in all US and some European extracts (Table 1), gives falsely high readings in the Lowry assay, and ninhydrin is such a sensitive method that it detects even protein molecules, too small to be immunologically active, resulting in falsely high readings when nondialyzed extracts are compared with dialyzed ones. However, the relative protein content—expressed as percentage of a lead extract assigned a 100% value—beats a quite close relation to the specific potency measurements, especially for the timothy grass pollen and cat extracts (Table 4).

Relative Potency Tests and Content of Major Allergens
Even though the reference sera and curves varied between the analyzing laboratories, quite consistent data are shown by the relative potency tests (Fig 2) and the determination of major allergens (Table 3). Evaluation of the performance of the different assay methods (Table 4) indicates that a good correlation can be found between the relative potency and the major allergen contents. For the Dermatophagoides pteronyssinus extracts, the best correlation coefficient is generated when the sum of the Der p 1 and Der p 2 content is plotted against the relative potency (AU/mL).

CONCLUSIONS
As can be deduced from these published results of the relative potency testing of several European SLIT maintenance solutions, the quantity of allergen given during maintenance SLIT treatment in Europe varies considerably among the different allergen manufacturers. However, apart from the precise allergen quantity delivered to the patient, other details may
Table 3. Cumulative monthly doses of SLIT maintenance therapy of four prominent European manufacturers

<table>
<thead>
<tr>
<th>Manu-facturers</th>
<th>D pteronyssinus (AU)a</th>
<th>Timothy (BAU)</th>
<th>Cat (BAU)</th>
<th>Short ragweed (AU)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Daily: 1,400</td>
<td>Monthly: 3,900</td>
<td>Daily: 5,100</td>
<td>Monthly: 12,600</td>
</tr>
<tr>
<td>Eur2</td>
<td>Daily: 1,050</td>
<td>Monthly: 42,000</td>
<td>Daily: 85</td>
<td>Daily: 3,550</td>
</tr>
<tr>
<td></td>
<td>Monthly: 1,000</td>
<td>Monthly: 5,500</td>
<td>Monthly: 2,550</td>
<td>Monthly: 106,500</td>
</tr>
<tr>
<td>Eur3</td>
<td>Daily: 100</td>
<td>Monthly: 3,800</td>
<td>Daily: 1,100</td>
<td>Monthly: 6,700</td>
</tr>
<tr>
<td></td>
<td>Monthly: 54,000</td>
<td>Monthly: 33,000</td>
<td>Monthly: 6,000</td>
<td>Monthly: 171,000</td>
</tr>
<tr>
<td>Eur4</td>
<td>Daily: 550</td>
<td>Monthly: 6,300</td>
<td>Monthly: 2,600</td>
<td>Monthly: 20,000</td>
</tr>
<tr>
<td></td>
<td>Monthly: 16,500</td>
<td>Monthly: 189,000</td>
<td>Monthly: 78,000</td>
<td>Monthly: 600,000</td>
</tr>
</tbody>
</table>

a As a reference: monthly probably effective doses recommended in US for SCIT are for house dust mite 500–2,000AU, timothy grass pollen 1,000–4,000BAU, cat 1,000–4,000 BAU (= 1.9–7.6 Fel d 1 U), and short ragweed pollen 1,000–4,000AU (6–12 Amb a 1 U). (1)

influence the efficacy of SLIT: the volume in which the dose is given (concentration), the stickiness of the solution (mucosal adhesives are under investigation), and other additives that might influence the reaction of the immune system. Furthermore, higher doses of SLIT lead also to a higher frequency of side effects, which might be different in different populations, and the compositions of US and European extracts vary, as we showed here specifically for house dust mite extracts. Therefore, the only way to truly prove the efficacy–safety balance of a sublingual allergen extract is to test each individual product in a clinical trial. Some SLIT trials are being undertaken in the United States, and major changes are underway in Europe, where the regulatory agencies are starting to require the manufacturers to show efficacy of immunotherapy extracts in clinical trials. Most of the European SLIT products had been registered until now as patient-named products. All these developments will likely lead to more specific dosing indications for allergen immunotherapy products.

ACKNOWLEDGMENTS
Jay E. Slater, MD, reviewed the first draft of this manuscript. We also thank other reviewers, who preferred to stay anonymous.

SUPPLEMENTARY DATA

REFERENCES


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PROTEIN CONTENT: SDS-PAGE PROTEIN PROFILES
A volume equivalent to 50 μg (or all of the sample if protein level was undetectable by assay) of buffer-exchanged sample was dried down by speed vac, and resolubilized in 200 μL of 2D sample buffer (9M urea, 4% CHAPS detergent, 30 mM dithiothreitol [DTT], 1.25% ampholytes). An 11-cm 3–10 pH immobile ph gradient isoelectric focusing strip (Bio-Rad Laboratories, Hercules, California) was rehydrated for 11 hours with each sample solution, and proteins were focused for 32,000 Vh. The strips were frozen at -80°C until ready for SDS-PAGE.

After thawing, the strips were equilibrated in 20% glycerol, 36% urea, 2% SDS, 62.5 mM Tris/Cl pH 8.8 with 1% DTT for 10 minutes (to be sure proteins are reduced), followed by 2% iodoacetamide (for alkylation of reduced proteins to prevent reoxidation) in the same buffer for 15 minutes. Strips were then positioned on top of 10.5–14% Criterion Tris/HCl precast SDS-PAGE gels (Bio-Rad Labs) and sealed in place with 1% melted agarose in running buffer. Precision protein standards (Bio-Rad Labs; 0.5 μL) was added to the single well on the acidic side of the strips. Gels were electrophoresed at 1 hour at 200 V, 15°C. After fixing overnight in 50% methanol/10% acetic acid, gels were washed and silver stained according to a modified method of Blum et al.38 Stain development time was the same within each allergen group, except that those samples with low protein load (lower than detection limit of protein assay) were allowed to develop until background started to darken to see as many spots as possible. Gels were scanned using PDQuest software and a GS800 Densitometer (Bio-Rad Labs). The pH gradient of the imaged gels is from left (acidic; pH 3) to right (basic; pH 10).

PROTEIN CONTENT, QUANTITATIVELY: DETAILS OF METHODS

Laboratory 1: Coomassie Plus (Pierce modified Bradford assay)

Laboratory 2: Bradford assay (Thermo Fisher Scientific) with bovine serum albumin as the standard

Laboratory 3: Ninhydrin Assay for Total Protein. Test samples and diluted bovine serum albumin standards that contain proteins are hydrolyzed to amino acids using 10 M sodium hydroxide and heat. Excess base is neutralized by the addition of concentrated hydrochloric acid. With the application of heat and a Ninhydrin solution (combination of methylcellulose, ninhydrin, citric acid, and stannous chloride), the amino acids react with Ninhydrin in a redox reaction to form a purple compound, which is quantified colorimetrically at 570 nm.

Laboratory 4: Lowry (BCA Assay Sigma Chemicals)

RELATIVE POTENCY TESTING: DETAILS OF THE METHODS FOR ELISA INHIBITION

Laboratory 1: Same Methods Used as in Laboratory 2. For the coating of the plates, FDA reference extracts were used of D pteronyssinus and Timothy grass pollen for the respective analyses.

Laboratory 2: For specific IgE competition ELISAs, reference D pteronyssinus or Timothy grass pollen extract was used to coat microplate wells (Costar, Lowell, MA) at approximately 2 μg/mL in carbonate coating buffer pH 9.6. Threefold serial dilutions of the test and reference extracts were prepared in phosphate buffer containing 0.05% Tween 20 (PBS-T), mixed with an equal volume of a 1:15 dilution of the reference human allergic serum pool, and added in duplicate to the respective allergen-coated microplate wells. After incubation at room temperature for 4 to 6 hours and washing the wells with PBS-T, the specific IgE bound was detected by overnight incubation of the wells with biotinylated anti-human IgE (Kirkegaard & Perry Laboratories, Gaithersburg, Maryland), followed by washing in PBS-T and alkaline-phosphatase–labeled avidin (Zymed Laboratories, San Francisco, California). After washing the plate and the wells in PBS-T, 1 mg/mL para-nitrophenyl phosphate substrate (Amresco, Solon, Ohio) was added to each well, and the absorbance was measured at 405 nm using a plate reader. The percent inhibition values were calculated for each sample dilution using the formula (Ap–As/Ap) × 100, where Ap is the average positive control (no inhibitor) and As is the average sample absorbance value. The best-fit dose–response lines containing at least 4 dilutions between 10% and 90% inhibition and bracketing 50% inhibition are constructed using linear regression analysis: y = a + bx, where y is the percent inhibition value, a is the y-intercept, b is the slope, and x is the logarithm to the base 3 of the dose. The best-fit parallel lines are computed using the combined data for the test and respective reference samples. For a valid assay, the corresponding coefficient for both the reference and test extracts must have been 0.95 or greater. Parallelism of the reference and test regression lines was checked using Student’s t-test, and the lines had to be parallel at P = .01. The log3 relative potency (W) was calculated using the following formula: W = (It–Ir)/B, where It and Ir are the test and reference y- intercepts calculated from the parallel lines, and B is the pooled slope. Three independent valid assays on the test antigen were used to calculate the relative potency (3W). The reference extract for D pteronyssinus had a potency of 10,000 AU/mL, and for Timothy grass pollen, 100,000 BAU/ml.

Laboratory 3: ELISA relative potency (Based on a CBER method). Extracts at various dilutions compete with immobilized allergens on microtiter plates for specific IgE obtained from several allergic donors. Bound IgE is determined using goat anti-IgE coupled to horseradish peroxidase and the enzyme substrate system TMB–hydrogen peroxidase. Uninhibited IgE produces the greatest color, and as more allergen inhibitor is mixed with the specific IgE, less color is generated. Potency of a sample is determined by comparing the regression line of the dose–response produced by the sample with that of a reference extract (ie, parallel-line bioassay method). Reference extract and sera are supplied by CBER. e-Table 1 lists the key parameters for each ELISA.
| e-Table 1. |
|-----------------|-----------------|
| **Timothy grass** | **Mite, *D pter** |
| **Plate coating** | **E8-Ti (100,000 BAU/mL), 1:600** | **E8-Dp (10,000 AU/mL)** |
| **Reference** | **E8-Ti (100,000 BAU/mL)** | **E8-Dp (10,000 AU/mL)** |
| **Serum** | **S5-GR** | **S5-Dpf** |

Laboratory 4: did not perform.
Laboratory 5: ELISA based on a CBER method for *D pteronyssinus* and timothy.

**RELATIVE POTENCY TESTING: DETAILS OF THE METHODS FOR RADIAL IMMUNODIFFUSION**

*Laboratory 1:* For SRagweed, the current FDA Amb a 1 RID was used. For cat, the major allergen content was determined using ALK-Abelló Fel d 1 ELISA, and the Fel d 1 Units were calculated: 1 Unit = 2.5 μg/mL Fel d 1. 
*Laboratory 2:* For radial immunodiffusion assays, dilutions of cat and SRagweed allergenic extracts were added to 3-mm-diameter wells, in quadruplicate, cut into 1% agarose plates containing reference anti-Fel d 1 serum. After incubating the plates for approximately 48 hours in a humidified chamber, the precipitin circles were visualized by dipping the plates in a 10% acetic acid solution for approximately 2 minutes. Alternatively, the precipitins were stained after exhaustive rinsing of the plates in distilled water followed by silver staining. The diameters of the precipitin circles were measured to the nearest 0.1 mm. The standard curves were generated using the labeled values of the Fel d 1 reference dilutions and the average diameter from quadruplicate wells. From these values, the best-fit regression line was calculated using the formula $y = a \log(x) + b$, where $y$ is average diameter in mm, $x$ is the labeled value of the specific antigen for that preparation in Fel d 1 units/mL, $a$ is the slope, and $b$ is the y-intercept. The correlation coefficient of the regression line must be greater than or equal to 0.9 for a valid assay. The average diameters of the of the test extract samples were calculated, and the Fel d 1 content was computed from the best fit regression line. 

*Laboratory 3:* Cat: (Based on a CBER method) This assay measures the quantity of Fel d 1 and Amb a 1 in allergenic extracts using radial immunodiffusion. For cat, a dose–response curve is determined from the diameters of precipitated circles in agar. Four dilutions of a reference preparation and a specific antiserum, which are provided by CBER, are used to generate the reference curve. From these data, a best-fit regression line is calculated. From the diameter of the circle produced by a test preparation, the concentration of Fel d 1 is calculated using the best-fit regression line of the standard dose–response curve. SRagweed polyclonal antisera, specific for Amb a 1, is mixed with dissolved agar and poured on a glass microscope slide or RID plate to solidify. The wells are then made in the agar, and the sample extract is added. The Amb a 1 will diffuse from the wells and interact with the polyclonal antibodies to form a precipitate ring. The diameter of this ring is directly related to the concentration of the Amb a 1 in the applied sample extract when compared with reference standards applied in the same manner. e-Table 2 lists the key parameters for the assay.

| e-Table 2. |
|-----------------|-----------------|
| **Lot number** | **Supporting information** |
| **Reference-Cat** | **C11-Cat** | Provided by CBER; 5, 10, 15, 20 units/mL. |
| **Antisera-Cat** | **S-2b** | Provided by CBER. |
| **Reference-SRW** | **C15-Ras** | Provided by CBER; 5, 10, 20, 30 units/mL. |
| **Antisera-SRW** | **RS9478** | Internal preparation of burro serum. |

SRW, short ragweed.
Laboratory 4: not performed.
Laboratory 5: radial immunodiffusion with the CBER recommended method for cat and short ragweed pollen.

**DETERMINATION OF MAJOR ALLERGEN CONTENT: DETAILS OF THE METHODS**

*Laboratory 1:* For the determination of contents of Der p 1 and Der p 2, laboratory 1 used the ALK-Abelló ELISA; similarly, for Timothy and cat, the ALK-Abelló Ph1 p 5 and Fel d 1 ELISAs were used. 

*Laboratory 3:* Assay kits for the determination of major allergens were purchased from Indoor Biotechnologies, Inc. (Charlottesville, VA). The assays are all direct sandwich ELISAs. In brief, monoclonal antibodies (mAb), which have been raised against the allergen of interest, are immobilized on microtiter plates. Antigen (in the reference or in samples) is added and binds to the captured mAb. A secondary, biotinylated mAb is added and binds to the immobilized antigen. Streptavidin-peroxidase and ABTS (with hydrogen peroxide) are used as the detection system. The responses of the samples are compared with a standard curve to determine the major allergen content of the sample. Four assay kits were used to determine the major allergen content of three extract types (Dpt, Timothy, and cat). e-Table 3 lists the key parameters for each ELISA. Neat samples were diluted so that the observed major allergen value was within the working range of the reference standards.
### e-Table 3.

<table>
<thead>
<tr>
<th>Extract Allergen</th>
<th>Mite, <em>D pter</em></th>
<th>Timothy grass pollen</th>
<th>Cat Fel d 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA kit item no. &amp; batch no.</td>
<td>EL-DP1 2905</td>
<td>EL-DP2 30032</td>
<td>EL-PP5 30016</td>
</tr>
<tr>
<td>Coating (capture) antibody</td>
<td>Anti-Der p 1 mAb Item No. MA-5H8 Lot No. 2760</td>
<td>Anti-Group 2 mAb Item No. MA-1D8 Lot No. 2760</td>
<td>Anti-Phl p 5 mAb Item No. MA-1D11 Lot No. 6001916</td>
</tr>
<tr>
<td>Reference Standard</td>
<td>Der p 1 Standard 2,500 ng/mL Item No. ST-DP1 Lot No. 2901</td>
<td>Universal Allergen Standard 2,500 ng/mL Item No. ST-UAS Lot No. 30006</td>
<td>Phi p 5 Standard 5,000 ng/mL Item No. ST-PP5 Lot No. 29083</td>
</tr>
<tr>
<td>Reference range</td>
<td>0.5–250 ng/mL</td>
<td>0.5–250 ng/mL</td>
<td>1–500 ng/mL</td>
</tr>
<tr>
<td>Secondary antibody</td>
<td>Biotinylated anti-Mite Group 1 mAb Item No. B1-4C1 Lot No. 2761</td>
<td>Biotinylated anti-Mite Group 2 mAb Item No. B1-7A1 Lot No. 2837</td>
<td>Biotinylated anti-Phi p 5 mAb Item No. B1-B01 Lot No. 29082</td>
</tr>
<tr>
<td>Detection system</td>
<td>Streptavidin-peroxidase and ABTS with H₂O₂</td>
<td></td>
<td></td>
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</tbody>
</table>