

# Standardization of House Dust Mite Extracts

## Liberation of Allergenic and Antigenic Components in Relation to Extraction Conditions

H. F. KAUFFMAN, S. VAN DER HEIDE, H. HOVENGA and K. DE VRIES

Dept. of Allergy, Clinic for Internal Medicine, State University Hospital, Groningen, The Netherlands

The spontaneous release of house dust mite components from cultures of *Dermatophagoides pteronyssinus* into slightly buffered water was studied against time, using both continuous and discontinuous extraction procedures. It was shown that proteins, carbohydrates, IgE binding components and precipitating antigenic components were rapidly released from the house dust mite cultures, reaching a maximal liberation within 1 h of extraction. Repeated extractions of house dust mite cultures (discontinuous extraction) showed an additional release of IgE components but the IgE binding potency declined after successive extractions, while showing increasing release of immunological inactive components. IgE binding to antigens immobilized to polystyrene surfaces (IgE-ELISA) appeared to be less sensitive compared with cyanogen-bromide activated discs (IgE-RAST). It was concluded that extraction procedures of house dust mite cultures with short incubation time of 1 h or less are to be preferred.

**Key words:** extraction time; IgE and IgG binding components; mite extracts.

*Accepted for publication 15 September 1984*

Allergen extracts of house dust mites as described in different studies show a wide variety of extraction procedures, either using spontaneous released antigens (1, 8, 14, 17, 19) or released after introduction of a destruction step (9, 13, 15). Furthermore, different times of extraction varying from 2 h till several days are described. Alternatively, mite bodies and excreta are separately studied (10, 17, 18). This wide variation in extraction conditions used makes it difficult to compare the results of the different studies and hampers the standardization of products used in clinical practice. Knowledge of the releasability of antigenic and allergenic components from the mite culture into the extraction medium as a function of time is limited. Tovey et al. (18) showed that one of the major allergens of the house dust mite *Dermatophagoides pteronyssinus* is released within 3 min from mite cultures, dust and

faeces. However, no systematic study has been done describing the rate of liberation of allergenic and antigenic components from house dust mite cultures into an extraction medium.

The aim of this investigation is to study the rate of liberation of allergenic (IgE-binding) material from the house dust mite, *D. pteronyssinus* into slightly buffered water. In order to discriminate between rapidly released components and components released after prolonged incubation times, discontinuous extraction conditions were also used next to continuous extractions.

## MATERIAL AND METHODS

House dust mite cultures (culture No. DP XVI) of *Dermatophagoides pteronyssinus* were kindly supplied by R. de Lange (Diephuis Laboratories, Groningen, The Netherlands).

Antigenic extracts were prepared by incubation (overhead rotation) of lyophilized mite cultures at 4°C with 0.01 M  $\text{NH}_4\text{HCO}_3$ , pH 8.6 (20 mg DPXVI/ml). When extraction periods exceeded 24 h, 0.1% azide was added to the incubation buffer in order to prevent microbial growth. Two extraction procedures were used:

1. Continuous extraction was performed by homogenization of a mite culture (100 ml) from which 10 ml samples were taken after different time intervals. The samples were centrifuged 10 min/4500  $\times g$  and the supernatant carefully removed;
2. Discontinuous extraction was performed by centrifugation of a house dust mite homogenate (10 ml) after a first incubation period of 1 h. After careful removal of the supernatant the pellet is reconstituted into a new 10 ml volume of extraction buffer. After the second incubation of 23 h, the homogenate is again centrifuged, supernatant removed and the pellet reconstituted in a same volume of extraction buffer and incubated for an additional period of 96 h.

The supernatants of all procedures were in part used for *direct* coupling to cellulose discs and polystyrene wells (volumetric immobilization of mite extracts) for monitoring the IgE binding potencies. The remainder of the supernatants were lyophilized and used for physical and chemical characterization and also for immobilization of extracts based on equal amounts of dry weight. The lyophilized extracts were stored at -20°C and used within 1 month for chemical and immunological characterization.

#### *Chemical and physical parameters*

The lyophilized material was used for measurement of the total amount of dry weight material, proteins and carbohydrates liberated during the extraction procedure.

Proteins were determined according to Lowry et al. (11). Carbohydrates were measured by the phenol-sulphuric acid method as described by McKelroy (12).

#### *Immunological procedures*

Sera used in this study are:

1. A positive pool serum of 45 patient sera known to contain high titres of IgE antibodies against *D. pteronyssinus*; (this pool serum was generally used diluted 1:4 with RAST buffer (Pharmacia) as indicated in the legend to the Figures);
2. A negative pool serum pooled from sera with negative RAST titres against house dust mite;
3. Hyperimmune rabbit serum: three rabbits were hyperimmunized with an extract of *D. pteronyssinus* (extract supplied by Diephuis Laboratories following an extraction period of 24 h under the conditions described above).

Hyperimmunization was performed as described previously for *Aspergillus fumigatus* (6), using 5 mg house dust mite extract/ml Complete Freund's Adjuvant. After 10 months of immunization the rabbits were bled and serum stored at -20°C.

#### *Immobilization of the mite extracts*

The IgE binding properties of the different extracts were studied by immobilization of the extracts to two carrier surfaces: cyanogen bromide-activated cellulose discs and polystyrene microtitre plates.

1. Activation and binding of the components to cyanogen bromide-activated cellulose discs was performed essentially according to the procedures described by Ceska & Lundkvist (2), using 0.5 ml of extract diluted to 2.5 ml with 0.1 M  $\text{NaHCO}_3$  (pH = 8.4)/10 activated discs. In some experiments immobilization was performed at 5 times lower antigen concentration, using 0.1 ml of extract/10 activated discs. This was done in order to control that differences in IgE binding potencies between different extracts were not masked by a maximal loading of the discs with less potent extracts.

- Spontaneous binding to microtitre plates as described for *Aspergillus fumigatus* (6) using 100  $\mu$ l mite extract/900  $\mu$ l sodium bicarbonate buffer 0.05 M, pH 9.6, containing 0.2%  $\text{NaN}_3$ . Wells of the microtitre plates (AR plates from Greiner) were sensitized with 250  $\mu$ l of diluted extract and incubated overnight at 4°C. Coating of residual binding sites was carried out with PBS, Tween, Gelatine buffer (7).

### Immunological parameters

Liberation of antigenic and allergenic material was studied by means of enzyme immunoassays.

The liberation of *IgE binding components* was studied using: 1) the enzyme allergosorbent test (EAST) with extracts immobilized to cellulose discs; and 2) the IgE-ELISA with extracts immobilized to microtitre plates.

Identity/non-identity of the IgE binding extracts was studied by means of the IgE-EAST inhibition.

IgE-EAST (Phadezym-RAST) was performed according to the instructions of the manufacturer (Pharmacia Diagnostics), and IgE-ELISA (plate procedure) was carried out as described before (4).

The negative pool serum and PBS-albumin buffer were used as controls. In order to study possible individual variations in IgE binding properties against the different extracts, IgE titres of different sera were measured separately.

### Inhibition studies

The procedure for IgE-EAST inhibition was similar to that of Le Mao et al. (9) for the RAST inhibition, with some modifications. Briefly: 50  $\mu$ l positive pool serum were added to 100  $\mu$ l volumes of mite extracts containing increasing amounts of antigen dissolved in RAST buffer (supplied with the Phadezym-RAST assay). After incubation for 3 h at RT the residual IgE was measured by the EAST assay using either immobilized antigen from the same extract (homologous inhibition) and/or from different extracts (heterologous or cross-inhibition).

Rocket-immunoelectrophoresis was carried out against hyperimmune rabbit serum on 6  $\times$  7 cm glass plates using 5% rabbit hyperimmune serum in the upper agarose phase and 8  $\mu$ l of mite extract (10 mg/ml distilled water). Electrophoresis was carried out for 5 h at 5 V/cm. Thereafter the slides were washed, dried and stained with Coomassie Brilliant Blue® (Serva) as described before (6).

## RESULTS

### Continuous extraction conditions

In Fig. 1 the amounts of total dry weight and protein extracted from a house dust mite culture are demonstrated, after different time intervals of continuous extraction. It is shown that 75% of the dry weight and protein are liberated within 1 h of extraction. A rapid

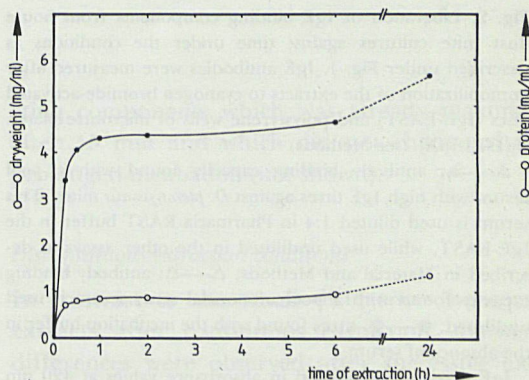


Fig. 1. Liberation of total amount of dry weight material and protein liberated from a house dust mite culture into an ammonium bicarbonate buffer as a function of time, using continuous extraction conditions.

Table 1

Protein, carbohydrates and residual dry weight material in house dust mite extracts in percentages of the total dry weight extracted after 1 and 24 h

| Time of extraction | Protein (%) | Carbohydrates (%) | Residual material (%) |
|--------------------|-------------|-------------------|-----------------------|
| 1                  | 18          | 26                | 56                    |
| 24                 | 21          | 25                | 54                    |

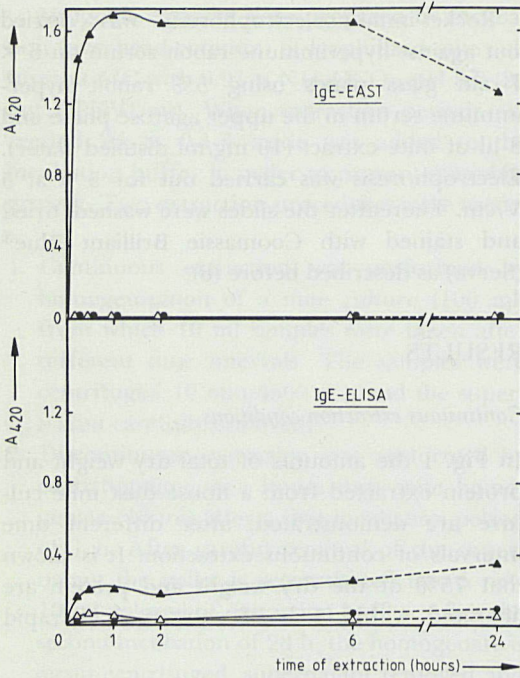


Fig. 2. Liberation of IgE binding components from house dust mite cultures against time under the conditions as described under Fig. 1. IgE antibodies were measured after immobilization of the extracts to cyanogen bromide-activated discs (IgE-EAST) and polystyrene wells of microtitre plates (IgE-ELISA), (see Methods).

▲—▲: antibody binding capacity found with a pool serum with high IgE titres against *D. pteronyssinus* mites. This serum is used diluted 1:4 in Pharmacia RAST buffer in the IgE-EAST, while used undiluted in the other assays as described in Material and Methods; △—△: antibody binding capacity found with a pool of normal sera; serum is used undiluted; ●—●: titres found with the incubation buffer in the absence of serum.

IgE titres are expressed in absorbance values at 420 nm ( $A_{420}$ ).

release of IgE binding components was found (Fig. 2), reaching a maximal value after 60 min of incubation with the IgE-EAST procedure. Longer incubation periods showed either similar or even lower titres (Fig. 2), except the IgE-ELISA titre that showed a slight increase after 24 h of extraction. It is important to note that the IgE titres measured by EAST are much higher when compared with titres of the ELISA, reflecting the higher antigen binding capacity of cyanogen bromide-activated cellulose discs (see Discussion). In addition to the

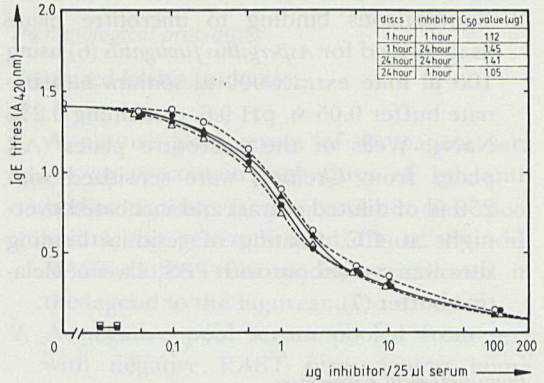


Fig. 3. Cross-inhibition of 1-h and 24-h extracts obtained after continuous extraction using IgE-EAST inhibition.

●, ○: 1 h extract immobilized to discs; ▲, △, 24 h extract immobilized to discs; closed symbols: homologous inhibition; open symbols: heterologous inhibition. □—□, ■—■: values obtained with the negative pool serum in the absence of inhibitor.

Conditions and 50% inhibition values (C50) are given in the inset. The positive pool serum was used undiluted using the conditions as described under Material and Methods.

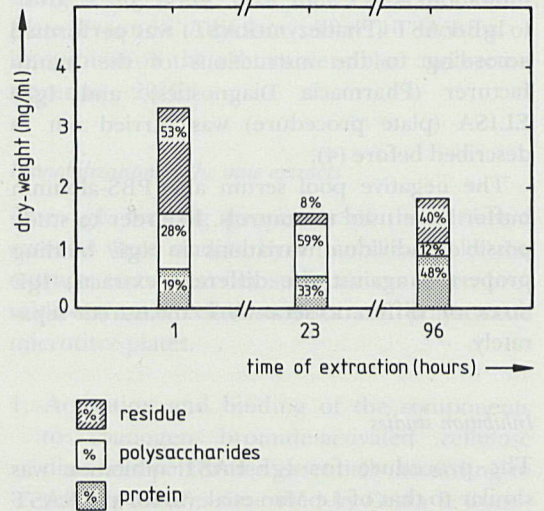


Fig. 4. Liberation of total amount of dry weight material, protein and carbohydrates after discontinuous successive incubation of 1, 23 and 96 h. Dotted bar: % of protein; open bar: % carbohydrates; shaded bar: % residual material.

high titres found by IgE-EAST, the binding in the presence of the IgE-negative pool serum and in the presence of buffer is very small, indicating a low aspecific binding of IgE antibodies.

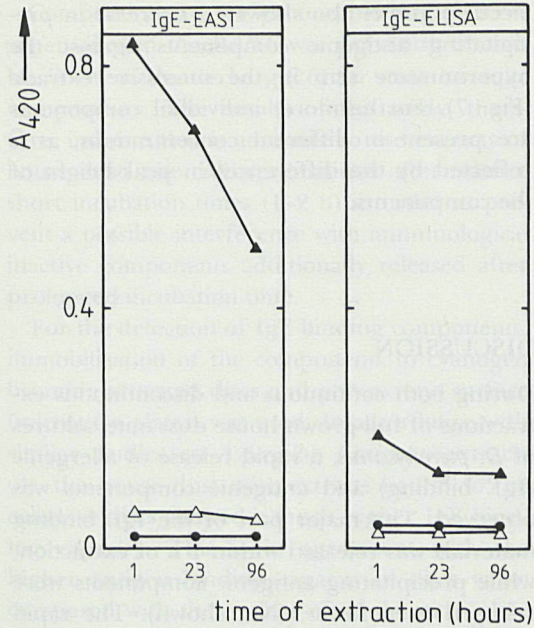


Fig. 5. IgE binding properties of house dust mite extracts obtained after discontinuous extractions as described under Material and Methods. IgE titres were measured as described in Fig. 2, and the Methods section.

▲—▲: antibody titres found with the positive pool serum; △—△: antibody titres found with the negative pool serum; ●—●: titres found with the incubation buffer in the absence of serum.

The positive pool serum is used diluted 1:9 in Pharmacia RAST buffer.

By contrast, the IgE-ELISA titres show a higher non-specific binding with both the negative pool serum and PBS-albumin buffer and a much lower binding of IgE antibodies with the positive pool serum. Cross-inhibition of 1 h and 24 h extracts by means of IgE-EAST as demonstrated in Fig. 3, shows similar inhibition curves for both homologous and heterologous conditions, indicating that the extracts have equal potencies.

The total inhibition found under these conditions indicates a total identity of the allergenic components from the 1 h and 24 h extracts.

In accordance with the rapid liberation of IgE binding components, rocket-immunoelectrophoresis of extracts after different incubation periods also showed a rapid release of precipit-

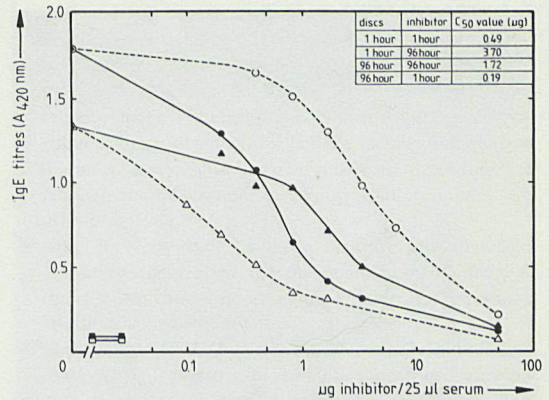


Fig. 6. Cross-inhibition of IgE binding potencies measured by IgE-EAST using the 1 h and 96 h extracts after the discontinuous extraction procedure.

●, ○: 1 h extract immobilized to discs; ▲, △: 96 h extract immobilized to discs; closed symbols: homologous inhibitions; open symbols: heterologous inhibitions. □—□, ■—■: values obtained with the negative pool serum.

The same extracts were used as described in Figs. 4 and 5, but lyophilized and immobilized using identical amounts of dry weight material (0.6 mg/10 discs). The positive pool serum is used undiluted.

ating components which was already maximal after 15 min and which did not change after prolonged incubation (not shown).

#### Discontinuous extraction conditions

In contrast to the small differences between extracts after continuous extraction, marked differences were observed after successive extractions for 1, 23 and 96 h. Fig. 4 shows that almost 50% of the extractable material (3.3 g) is released after 1 h of incubation, while, in addition, 1.6 and 1.8 g were released after 23 and 96 h incubations, successively. The concentrations of proteins and polysaccharides also differ between the extracts, showing increased liberation of proteins with longer incubation periods.

The IgE binding properties as measured by IgE-EAST decrease markedly after the successive extractions (Fig. 5) indicating that the major part of the IgE binding components was released after 1 h of extraction. The same conclusion can be drawn from the ELISA-IgE titres but, in accordance with the observations de-

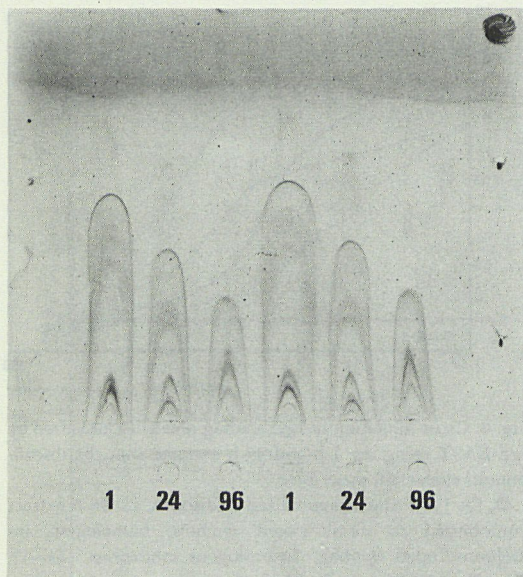


Fig. 7. Rocket-electrophoresis of the 1, 23, and 96 h extracts of the discontinuous extraction procedure, using hyper-immune rabbit serum. Measurements were performed in duplicate.

scribed under Fig. 2, titres obtained with this procedure are much lower.

In accordance with the differences found in the total IgE binding potencies between the extracts of the discontinuous procedure, cross-inhibition between the 1 h and 96 h extract also showed differences with volumetric coupled discs (not shown). Since the 1 h and 96 h extracts contained smaller amounts of dry weight materials after prolonged incubations (see Fig. 4), inhibition studies were also performed with discs loaded with equal amounts of dry weight material (Fig. 6). Inhibition studies with the "equal dry weight discs" (Fig. 6) show that the allergenic potency of the 1 h extract is higher both in the homologous and heterologous inhibitions ( $C_{50} = 0.5 \mu\text{g}$  and  $0.3 \mu\text{g}$ , resp.) compared with the inhibitory potency of the 96 h extract ( $C_{50} = 2.7$  and  $3.7 \mu\text{g}$  for homologous and heterologous inhibition, resp.).

These latter experiments indicate that fewer IgE binding components are released per mg of dry weight extract in the 96 h extract compared with the 1 h extract. In accordance with the results described above (Figs. 4–6), rocket-

electrophoresis also showed a decrease in precipitating antigenic components against the hyperimmune sera in the successive extracts (Fig. 7). Furthermore, individual components are present in different concentrations, as is reflected by the differences in peak-height of the components.

## DISCUSSION

During both continuous and discontinuous extractions of full-grown house dust mite cultures of *D. pteronyssinus*, a rapid release of allergenic (IgE binding) and antigenic components was observed. The major part of the IgE binding materials was released within 1 h of extraction, while precipitating antigenic components were released even faster. (Not shown). The rapid liberation of allergenic components is in accordance with the fast release of the major allergen  $P_1$  as was shown by Tovey et al. (18). During prolonged incubation (continuous extraction) additional material (dry weight) containing proteins and carbohydrates was released. Although additional release was observed, the IgE binding properties remained similar (Fig. 3). By contrast, house dust mite cultures that were repeatedly extracted (discontinuous extraction) showing liberation of more material (dry weight), also showed additional IgE binding capacities (Fig. 5). This observation indicates that allergenic components released during the first hour of incubation may prevent the release of additional IgE binding components during conditions of continuous extraction. The materials additionally released after discontinuous extraction showed a lower IgE binding potency, both per ml extract and per mg of dry weight material (Fig. 6), indicating an increasing release of immunological inactive components. The release of immunological inactive components after prolonged incubations might interfere and compete with the binding sites on the paper discs or polystyrene surface. This could also explain the fall in IgE binding capacity of the 24 h extract after continuous extraction (Fig. 2).

Competition for the protein binding sites on paper discs in the RAST was previously shown by Tovey & Vanderberg (16) for some *non*-mite components. The results of this study, therefore, indicate that is advisable to use extracts of house dust mite cultures obtained after relative short incubation times (1–2 h) in order to prevent a possible interference with immunological inactive components additionally released after prolonged incubation time.

For the detection of IgE binding components, immobilization of the components to cyanogen bromide-activated discs and polystyrene surface (microtitre plates) was used. In accordance with similar studies on *Aspergillus fumigatus* extracts (4), the house dust mite extracts bound to the cellulose discs showed a much higher IgE binding capacity. This is in agreement with the higher antigen binding capacity of discs when compared with the surface of a microtitre plate (3, 5). The lower antigen binding capacity of the polystyrene surface for the IgE binding components may result in an excess of IgG and IgE antibodies competing for the relatively small numbers of allergens bound to the surface (20). Therefore the spontaneous immobilization of house dust mite extracts to polystyrene surfaces is less suitable for the measurements of concentrations of IgE antibodies in patient sera.

## ACKNOWLEDGEMENTS

We would like to thank Diephuis Laboratories for their support and generous supplies of *D. pteronyssinus* culture. We thank Mrs. J. Kruitbosch-Koopmans for her expert secretarial help.

## REFERENCES

- Berrens, L., Bronswijk, J. E. M. H. van, Young, E. & Dijk, A. G. van: A controlled study of allergen production in cultures of *Dermatophagoides pteronyssinus*. *Acta Allergol.* 30, 390–410, 1975.
- Ceska, M. & Lundkvist, V.: A new and simple radio-immunoassay method for the determination of IgE. *Immunochemistry* 9, 1021–1030, 1972.
- Hamilton, R. G., Hussain, R., Ottesen, E. A. & Adkinson, N. F., Jr.: The quantitation of parasite-specific human IgG and IgE in sera: evaluation of solid-phase RIA and ELISA methodology. *J. Immunol. Methods.* 44, 101–114, 1981.
- Heide, S. van der, Kauffman, H. F. & Vries, K. de: Measurement of IgE and IgG antibodies against *Aspergillus fumigatus* antigen in patients' sera, using enzyme immuno assays. Influence of different procedures of antigen immobilization. *J. Allergy Clin. Immunol.* 73, 813–818, 1984.
- Hill, P. N. & Liu, F. T.: A sensitive enzyme-linked immunosorbent assay for the quantitation of antigen-specific murine immunoglobulin E. *J. Immunol. Methods* 45, 51–63, 1981.
- Kauffman, H. F. & Vries, K. de: Antibodies against *Aspergillus fumigatus*. II. Identification and quantification by means of crossed immunoelectrophoresis. *Int. Arch. Allergy Appl. Immunol.* 62, 267–275, 1980.
- Kauffman, H. F., Beaumont, F., Meaurs, H., Heide, S. van der & Vries, K. de: Comparison of antibody measurements against *Aspergillus fumigatus* by means of double-diffusion and ELISA. *J. Allergy Clin. Immunol.* 72, 255–261, 1983.
- Kerrebijn, K. F., Degenhart, H. J. & Hammers, A.: Relation between skin tests, inhalation tests, and histamine release from leukocytes and IgE in house dust mite allergy. *Arch. Dis. Child.* 51, 252–258, 1975.
- Le Mao, J., Weyer, A., Pauli, G., Lebel, B. & David, B.: Studies on *Dermatophagoides pteronyssinus* allergens: measurement of the relative potencies of *D. pteronyssinus* purified extracts by in vitro and in vivo methods. *J. Allergy Clin. Immunol.* 65, 381–388, 1980.
- Lind, P.: Standardization of mite extracts. Qualitative and quantitative investigation of three kinds of preparations with CRIE and RAST. *Allergy* 35, 227–230, 1980.
- Lowry, O. H., Rosebrough, N. H., Farr, A. L. & Randall, R. J.: Protein measurement with the Folin-phenol reagent. *J. Biol. Chem.* 193, 265–275, 1951.
- McKelvy, J. F. & Less, Y. C.: Microheterogeneity of the carbohydrate group of *Aspergillus oryzae*  $\alpha$ -amylase. *Arch. Biochem. Biophys.* 132, 99–110, 1969.
- Pauli, G., Bessot, J. C., Hirth, C. & Theirry, R.: Dissociation of house dust allergies; a comparison between skin tests, inhalation tests, specific IgE, and basophil histamine release measurements. *J. Allergy Clin. Immunol.* 63, 245–252, 1979.
- Spiekma, F. T. M. & Voorhorst, R.: Comparison of skin reactions to extracts of house dust mites and human skin scales. *Acta Allergol.* 24, 124–146, 1969.
- Stewart, G. A. & Turner, K. J.: Physicochemical and immunochemical characterization of the allergens from the mite *Dermatophagoides pteronyssinus*. *Austr. J. Exp. Biol. Med. Sci.* 58, 259–274, 1980.
- Tovey, E. R. & Vandenberg, R. A.: Effects of reagents and allergen extracts on radioallergosorbent assays for mite allergen. *Clin. Allergy* 8, 329–339, 1978.
- Tovey, E. R. & Vandenberg, R. A.: Mite allergen content in commercial extracts and in bed dust determined by radioallergosorbent test. *Clin. Allergy* 9, 253–262, 1979.
- Tovey, E. R., Chapman, M. D. & Platts-Mills, T. A. E.: IgG and IgE in sera: evaluation of solid-phase RIA and ELISA methodology. *J. Immunol. Methods.* 44, 101–114, 1981.

Mite faeces are a major source of house dust allergens. *Nature* 289, 592-593, 1981.

19. Warner, J. O.: Significance of late reactions after bronchial challenge with house dust mite. *Arch. Dis. Child.* 51, 905-911, 1976.
20. Zimmerman, E. M., Yunginger, J. W. & Gleich, G. J.: Interference in ragweed pollen and honey bee venom radioallergosorbent tests. *J. Allergy Clin. Immunol.* 60, 386-393, 1980.

Address:

Henk F. Kauffman, Ph.D.  
Department of Allergology  
Clinic for Internal Medicine  
State University Hospital  
Oostersingel 59  
9713 EZ Groningen  
The Netherlands

This document is a scanned copy of a printed document. No warranty is given about the accuracy of the copy. Users should refer to the original published version of the material.