ARQUIVOS DE ASMA, ALERGIA E IMUNOLOGIA


*Original Article*

# Study of protein profile and immunochemical reactivity for extracts of *D. farinae*, *D. pteronyssinus* and *B. tropicalis* mites in the city of Rio de Janeiro, Brazil

*Estudo do perfil proteico e reatividade imunoquímica dos extratos dos ácaros* D. farinae*,* D. pteronyssinus *e* B. tropicalis *na cidade do Rio de Janeiro, Brasil*

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**ABSTRACT**

**Objective:** In tropical countries like Brazil, the mapping of protein constituents of mite extracts becomes relevant in the context of therapeutic evaluations of respiratory allergic diseases. In addition, this allows to set up an internal national database of the population's sensitization profile. Extracts are complex mixtures containing different proportions of biological materials, and the determination of their protein profile is necessary for an improved performance in diagnosis and therapy. Therefore, in this study, we used electrophoresis to characterize the protein constituents of *B. tropicalis*, *D. farinae* and *D. pteronyssinus* extracts, and evaluated the reactivity of specific immunoglobulins from atopic volunteers from the city of Rio de Janeiro, Brazil. **Methods:** Samples of the extracts were subjected to precipitation with acetone and applied to polyacrylamide gel 12.5%. After the electrophoretic run, samples were transferred to a 0.45 µm nitrocellulose membrane, followed by incubation with the serum pool. Bands related to antigen-antibody reactivity were obtained by the colorimetric method, using peroxidase conjugated to secondary antibody. **Results:** The results obtained showed that protein precipitation favored the visualization of bands in the polyacrylamide gel; homogeneity was evidenced by experiment reproducibility and low standard deviation values. With regard to electrophoretic profile, the extracts showed specific proteins in their composition, with positive reactivity to the immunochemical test. **Conclusion:** One may infer that mite extracts in Brazil present different allergens when compared to extracts described in the American and European literature, warranting the development of new, specific criteria for extract standardization.

**Keywords:** Dust, immunotherapy, alergens.

**RESUMO**

**Objetivo:** Em países tropicais, como o Brasil, o mapeamento dos constituintes proteicos de extratos de ácaros torna-se informação relevante quando se pretende fazer avaliações terapêuticas de doenças alérgicas respiratórias, além de contribuir para a construção de um banco de dados interno do país. Os extratos apresentam-se como misturas complexas contendo diferentes composições de material biológico, sendo necessária a determinação do seu perfil proteico para um melhor desempenho em diagnóstico e terapia. Assim, este estudo analisou a eletroforese das proteínas constituintes dos extratos de *B. tropicalis*, *D. farinae* e *D. pteronyssinus* e avaliou a reatividade das imunoglobulinas específicas de soros de voluntários atópicos da cidade do Rio de Janeiro, Brasil. **Métodos:** Amostras dos extratos foram submetidas a precipitação com acetona e aplicadas em gel de poliacrilamida 12,5%. Após a corrida eletroforética, as amostras foram transferidas para membrana de nitrocelulose de 0,45 µm, seguido da etapa de incubação com *pool* de soros. As bandas referentes à reatividade antígeno-anticorpo foram obtidas pelo método colorimétrico, utilizando-se a peroxidase conjugada a anticorpo secundário. **Resultados:** Os resultados obtidos mostraram que a precipitação proteica favoreceu a visualização das bandas no gel, mostrando sua homogeneidade pela reprodutibilidade dos experimentos e seu baixo desvio padrão. Também quanto ao perfil eletroforético, os extratos apresentaram proteínas específicas em sua constituição, tendo reatividade positiva ao teste imunoquímico. **Conclusão:** Pode-se inferir que os extratos de ácaros obtidos no Brasil apresentam alérgenos diferentes quando comparados com extratos proteicos apresentados na literatura americana e europeia, o que torna necessária a criação de critérios próprios de avaliação para padronização dos extratos.

**Descritores:** Poeira, imunoterapia, aérgenos.

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## Introduction

Mite species *Dermatophagoides farinae* (DEF), *Dermatophagoides pteronyssinus* (DEP) and *Blomia tropicalis* (BT) are the main triggering agents of allergic respiratory processes of atopic diseases such as allergic rhinitis, allergic conjunctivitis, dermatitis and allergic asthma.[1-4] They are induced by inhalation of allergenic substances usually present in dust particles at home (bed, sofa, toys) and in general dirt in the houses.[5,6] A large number of studies have consistently shown major allergens secreted in the feces and present in the body composition of these organisms. For the two *Dermatophagoides* species, major allergens include those with a molecular weight of 25 kDa, hereinafter referred to as Der f 1 and Der p 1, and others with about 15 kDa, corresponding to Der f 2 and Der p 2.[7-10] For *B. tropicalis*, the major known allergen sensitizers are Blo t 1 (25 kDa), homologous to Der f 1 and Der p 1, with 60-90% reactivity of specific IgE antibodies, and Blo t 2 (14 kDa), with 63% reactivity of specific IgE. There are literature reports on cross-reactivity among allergens from *B. tropicalis* with *D. farinae* and *D. pteronyssinus*.[11,12] Other allergens that are specific to *B. tropicalis* have been investigated.[13-15] Allergenic proteins are studied according to their immunological characteristics and can be determined from preparation of mite extracts and their further characterization by electrophoresis and immunoblotting.[16-18]

In the stage of protein characterization of extracts, variations may occur in the protein profile due to changes in the amino acid sequence. These variables are strongly influenced by temperature, humidity and feed, as the metabolic genetics of these organisms is different even within the same species.[14,15,19,20] Changes in protein composition of the allergens present in the extracts can directly influence the efficacy of treatment of atopic patients. The need for allergen identification and characterization is relevant in the context of vaccine production and marketing for use in diagnosis and immunotherapy. In this sense, electrophoresis in polyacrylamide gel has been a widely employed technique for protein identification along with the immunoblotting. This study aims to investigate and characterize the major allergens present in *D. farinae*, *D. pteronyssinus* and *B. tropicalis* extracts obtained from house dust in the city of Rio de Janeiro, Brazil, and to evaluate the reactivity to specific IgE antibodies in the sera of atopic patients.

## Methods

### *Selection of individuals and skin test*

Volunteers diagnosed as atopic agreed to participate in the study and authorized the collection of blood through a standard protocol. A total of 15 serum samples were obtained from participants with a prick test positive for *B. tropicalis*, *D. farinae* and *D. pteronyssinus*. Blood samples were centrifuged for serum separation in a centrifuge (Anti-Celm) at 1118 xg for 20 min. Sera were stored at -20 °C in aliquots until the immunochemical test.

### *Allergen extract preparation*

Samples containing the mites under study were collected in households in the city of Rio de Janeiro, Brazil, by mechanical aspiration (portable vacuum, NV3600, Black&Decker) from beds, sofas and tapestry. After collecting the powder, larger debris were separated using an 80-mesh sieve. Mites of the species of interest (*B. tropicalis*, *D. farinae*, *D. pteronyssinus*) were isolated using a stereoscopic microscope (Option Microscope, TNE-10BN), considering its taxonomic classification, according to the literature[21]. The obtained mass of each mite species was cultivated artificially in containers with suitable feeding (fish feed) at 25 °C. The containers were covered by mesh screen. Mites were cultivated by approximately 90 days. At the end of this time, mites were isolated from debris using a 400-mesh sieve, and the final mass was obtained, containing the mites' bodies and feces; this mass was used for the preparation of extracts.

Extracts were prepared as described by Lima & Da Silva,[22] with modifications. A solution of 10% (w/v) of mites (body and feces) diluted in pH 7.0 saline buffer was homogenized using a sonicator (Ultrasonic Concertot), at a frequency of 20 kHz cell disruption for 20 min. Then, the homogenate was alkalinized with sodium hydroxide 2.0 mol/L$^{-1}$ to pH 8.5. After homogenization, the extract was allowed to stand at 4 °C for 48 h and then centrifuged at 1118 xg for 30 min at 25 °C. The supernatant was filtered using a 0.22-µm pore-size membrane and glycerol was added at the rate of 40% (v/v) for the purposes of stability, finally adjusting the pH to 7.0.

### Concentration of proteins by acetone precipitation

Protein extracts were concentrated according to the methodology described by Thermo Fisher Scientific.[23] The precipitant agent used was acetone chilled to -20 °C, which was added in excess at 3:1 (acetone:extract) with successive dripping, keeping the system cooled next to 0 °C for 30 min. After addition of 10 mL of acetone, the mixture showed turbidity and was centrifuged at 1118 xg for 30 min at 25 °C, aiming at separation of the supernatant from the pellet. The precipitate was resuspended in 200 µL of distilled water and stored at -20 °C.

### *Protein dosage*

Protein concentration of the mite extracts was performed according to the modified Lowry method[24]. At first, a calibration curve was plotted from successive dilutions of a 1.0 mg.mL$^{-1}$ bovine serum albumin (BSA) standard sample, from 0.01 to 0.07 mg/mL$^{-1}$. Samples were diluted as necessary in injectable grade water. To an aliquot of 200 µL BSA were added 800 µL of distilled water, 3.0 mL of sodium carbonate 1%, 0.5 mL of copper sulfate 0.1%; after homogenization, samples were allowed to stand for 10 min at 25 °C. Then 500 µL of Folin-Ciocalteu (Sigma-Aldric, Missouri, USA) reagent previously diluted at 1:10 was added in a test tube and left to stand at dark for 30 min. Absorbance readings were performed using an ultraviolet-visible spectrophotometer (Spectrophotometer 1102, Bel Photonics, Brazil) at a wavelength of 625 nm. The protein content was calculated by the equation of the calibration curve, considering the correlation coefficient.

### *Polysaccharide dosage*

The concentration of polysaccharides was determined using dinitrosalicylic acid (DNS) according to Ferreira's[25] modified protocol. Briefly, a calibration curve with standard glucose solution ranging from 0.1 to 1.0 mg/mL$^{-1}$ was plotted. An aliquot of 1.0 mL was taken and added to 1.0 mL of DNS reagent, and boiled at 100 °C for 5 min. After cooling, 16.0 mL of potassium sodium tartrate 0.2 mol/L$^{-1}$ was added and the readings were done in the ultraviolet-visible spectrophotometer at a wavelength of 540 nm. The polysaccharide content was calculated by the equation of the calibration curve, considering the correlation coefficient.

### *Polyacrylamide gel electrophoresis*

Protein characterization of *D. pteronyssinus*, *D. farinae* and *B. tropicalis* extracts was performed by electrophoresis in denaturing conditions (SDS-PAGE 12.5%) and by adding sodium dodecyl sulfate (SDS) and 2-mercaptoethanol. A vertical electrophoresis system (Mini-Protean Tetra, Bio-Rad, California, USA) was used. Experiments were monitored and evaluated for repeatability and homogeneity of the protein composition of the extracts. One single experiment was carried out under non-denaturing conditions (*in natura*) according to the methodology described by Ornstein and Davis,[26] with modifications. For electrophoresis, the resolving gel was prepared in a Tris-HCl-SDS 0.75 mol/L$^{-1}$ buffer solution, with 0.2% SDS and pH 8.8, and the stacking gel in a Tris-HCl-SDS 0.25 mol/L$^{-1}$ buffer, with 0.2% SDS and pH 6.8. The buffering electrolyte of pH 8.3 used for electrophoresis was prepared by solubilizing Tris-glycine 5 mM (pH 8.6) containing 0.1% SDS.

Samples were prepared by adding 40 µL of mite extracts and 40 µL of reducing buffer containing 0.1 M Tris-Cl, pH 8.45, 1% (w/v) SDS, 2% (v/v) 2-mercaptoethanol, 20% (w/v) glycerol, and 0.04% bromophenol blue at the absence of reducing agents in the *in natura* sample. All reagents used had a high degree of purity and were purchased from Sigma-Aldric (Missouri, USA). The molecular mass (kDa) of samples was estimated by plotting a standard curve of protein (PageRuler/Bio-Rad) from measurements of the distances traveled by the marker proteins vs. the logarithms of their molecular weights, using the software GelAnalyzer 10a. The apparent molecular mass of the allergens was estimated using the marker protein standards of the PageRuler Unstained Broad Range Protein Ladder (Thermo Scientific- Waltham, Massachusetts, USA) and Plus Protein™ Dual Xtra Standards (Bio-Rad, California, USA).

### *Immunoblotting*

Sera from 15 mite allergic patients in the study were used in antigen-antibody reactivity tests (IgE). The proteins from extracts, separated by SDS-PAGE electrophoresis on 5 to 12.5% gradient gel were transferred to a 0.45-µm pore-size nitrocellulose membrane (Bio-Rad, California, USA). The membrane and the gel containing the allergens were placed in sandwich form, covered with filter paper and compressed with polyurethane sponges on the transfer support. All material was previously soaked in saline

buffer, pH 7.2, comprising 25 mM Tris Base, 192 mM glycine and 20% methanol. Slow transfer conditions were applied with constant amperage of 10 mA, 18 V and 70 W for 18 h at 4 °C. The transfer of proteins from the gel to the membrane was confirmed by gel staining with silver nitrate and membrane staining with Ponceaus S (Sigma-Aldric, Missouri, USA). After the transfer, the membrane was incubated in blocking solution containing BSA 3% in TBS saline buffer for 30 min. Then, the membrane was washed with saline buffer containing 0.1% Tween (TBS-T) and incubated for 12 h in solution containing primary antibody, consisting of the pool serum (IgE) diluted at 1:10. Finally, the membrane was washed with TBS-T buffer and received secondary antibody (goat anti-human; Sigma-Aldric, Missouri, USA) conjugated to peroxidase enzyme at 1:200 dilution for a period of 4 h. Antigen-antibody binding reactivity was determined by a blue color development, provided by reaction of the developing solution containing the substrate of peroxidase and 4-chloro-1-naphtol (Amplified Opti-4CN Kit; Bio-Rad, California, USA).

## Results and discussion

### *Total protein and carbohydrate determination*

For mite extract samples that passed the precipitation process, an attempt was made to increase the resolution of bands in both electrophoresis and immunoblotting, by increasing the protein load. The protein content was determined by repeating five independent analyses, as seen in Table 1.

The precipitation step was effective in increasing the concentration of proteins in the extracts. This process can also be used as a device to remove impurities. Regarding the sugar content, carbohydrate concentrations were 0.578 mg.mL$^{-1}$ for *B. tropicalis*, 0.727 mg.mL$^{-1}$ for *D. farinae* and 1.375 mg.mL$^{-1}$ for *D. pteronyssinus*. Generally, in mite extracts, several proteins and glycoproteins are present, with epitopes arranged in carbohydrate chains, which may justify the concentrations found in the prepared extracts and the positive reactivity with high molecular weight proteins as shown in the blot results (Figure 1).

### *SDS-PAGE for mite extracts*

The preparation of polyacrylamide gels at 12.5% was efficient for the separation and presentation of the protein profile of mite extracts. The presence of proteins known and characterized in the literature was confirmed and identified by electrophoretic separation. Specific proteins in the extracts obtained from the cultivation of mites were also observed (Figure 2).

As shown in Figure 2, after staining with silver nitrate of the three mite extract proteins with molecular weight at about 28-30 kDa, hypothetical allergen known as Trypsin was found to link to serine protease enzyme. According to Wayne et al.,[21] this protein shows a percentage of 16-100% binding to IgE in studies with subjects sensitized by *B. tropicalis*, *D. farinae* and *D. pteronyssinus*. Some lipids also linked to protein, as revealed by the presence of two bands at about 14-17 kDa for *Dermatophagoides*. *D. farinae* extract precipitation with acetone was effective to

**Table 1**
Total protein concentration in the mite extracts

| Extract | Protein determination (mg.mL$^{-1}$) | | | | | $\bar{x}$ [a] | $\delta$ [b] | $S_x$ [c] |
|---|---|---|---|---|---|---|---|---|
| $BT_{(CE)}$ | 1.301 | 1.444 | 1.444 | 1.301 | 1.372 | 0.071 | 0.014 | 1.372 |
| $BT_{(ppt)}$ | 2.535 | 7.606 | 7.748 | 7.748 | 7.462 | 0.110 | 0.023 | 7.634 |
| $DEF_{(CE)}$ | 0.708 | 0.730 | 0.730 | 0.730 | 0.618 | 0.048 | 0.009 | 0.703 |
| $DEF_{(ppt)}$ | 1.444 | 1.515 | 1.587 | 1.444 | 1.372 | 0.170 | 0.034 | 1.412 |
| $DEP_{(CE)}$ | 0.416 | 0.460 | 0.438 | 0.438 | 0.438 | 0.015 | 0.003 | 0.438 |
| $DEP_{(ppt)}$ | 0.786 | 0.741 | 0.741 | 0.741 | 0.741 | 0.020 | 0.004 | 0.750 |

[a] Mean, [b] standard deviation, [c] standard error. BT = *B. tropicalis*, DEF = *D. farinae*, DEP = *D. pteronyssinus*, CE = crude extract, ppt = precipitate extract.

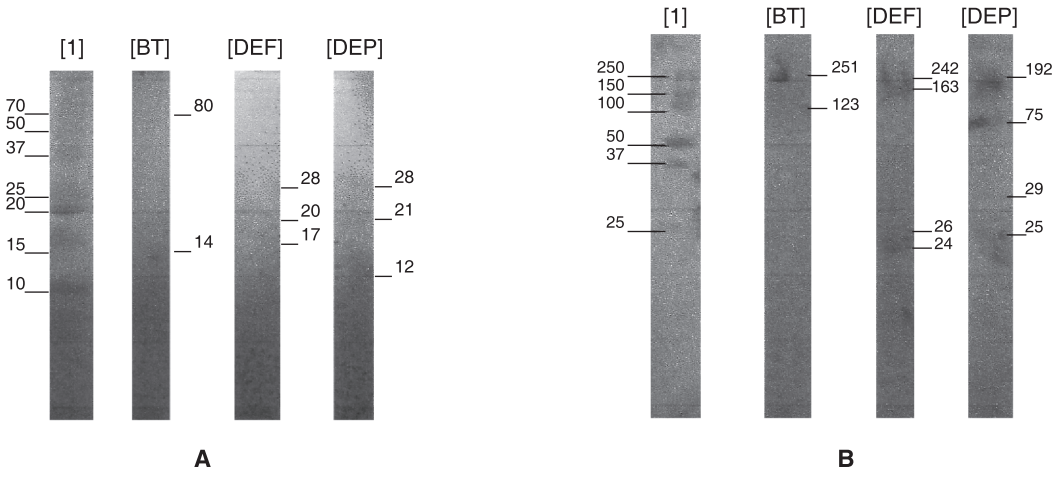


**Figure 1**
Immunoblot in 0.22 µm nitrocellulose membrane. Reactivity of the sera of 15 individuals allergic to mites. [BT] *B. tropicalis*, [DEF] *D. farinae* and [DEP] *D. pteronyssinus* for denatured sample [A]; [B] *in natura* sample and [1] Plus Protein™ Dual Xtra Standards (Bio-Rad, California, USA)

concentrate proteins, as seen in Figure 2 [C] and [Cp], favoring the appearance of strong bands near 14 and 29 kDa.

The *B. tropicalis* extract is known to have impurities that may be seen as drags and tend to precipitate together with proteins[27] (Figure 2B), even after dilution attempts in the range 1:100 to 1:10. In this case, the obtained extract must be subjected to a more efficient purification system, such as column chromatography. Despite the protein content (0.293 mg.mL$^{-1}$) present in the *B. tropicalis* extract, its electrophoretic profile failed to show strong bands; rather, only one visible band near 25 kDa was observed. Other bands were identified, but with mild intensity, as shown in Table 2, obtained with five replicate analysis of SDS-PAGE gels. The band displayed on the gel with molecular weight next to 64-70 kDa is characteristic of this cultivated mite, but references in the literature were not found. The protein of 101 kDa may be characterized as the hypothetical allergen Blo t 11 (paramyosin), and that of 35 kDa, as Blo t 10 (tropomyosin).[28] Blo t 11 sensitization is present in about 50% of individuals studied by Branch et al.[29] Santos et al. conducted a survey in the city of São Paulo, Brazil, and showed tropomyosin as a high-affinity IgE allergen with cross-reactivity with other mites[30].

The repeatability of protein content analysis in the gels within a short period of time showed correlated results. There was little dispersion among repetitions of the same protein bands after preparation of different gels, evidenced by the low standard deviation and variation coefficient obtained. Tables 2, 3 and 4 show the molecular weight in kDa of the major proteins found in the extracts precipitated with acetone.

### *Immunoblotting*

In both techniques employed (*in natura* and denatured), extracts showed positive reactivity to the specific immunoglobulin (IgE) present in the serum of allergic patients and to immobilized proteins on the surface of the membrane extracted from mites, as shown in Figure 1.

Regarding the protein profile obtained by electrophoresis and immunoblotting (Figure 1), it can be inferred that there are characteristic, unique proteins in the extracts, which have reacted positively to the specific immunoglobulin (IgE) present in the serum of patients sensitized to mites. The proteins that showed positive reactivity in the immunochemical test, with molecular weights of 251 kDa, 123 kDa, 242 kDa,

163 kDa, 192 kDa and 75 kDa, may trigger allergic reactions in atopic individuals, even though most of them are not cited in literature[27].

Allergens present in mite extracts in Brazil may differ from those observed in other countries. Jeong et al.[18] compared commercial mite extracts from Korea and United States used in immunotherapies. The authors reported differences in the allergen content present in the extracts, and alerted for the need to adapt the composition of extracts in each country for use in diagnosis and therapy.

## Conclusion

Through the established method of protein extraction and characterization and upon confirmation by IgE-reactivity profile specific to allergens in the immunochemical test, we confirmed the efficiency of using *D. farinae*, *D. pteronyssinus* and *B. tropicalis* extracts for the purposes of application in diagnosis and therapy. Further studies in tropical countries are warranted, with other allergens of interest, contributing to the creation of a data base and thus improving the treatment of several atopic diseases in our country.

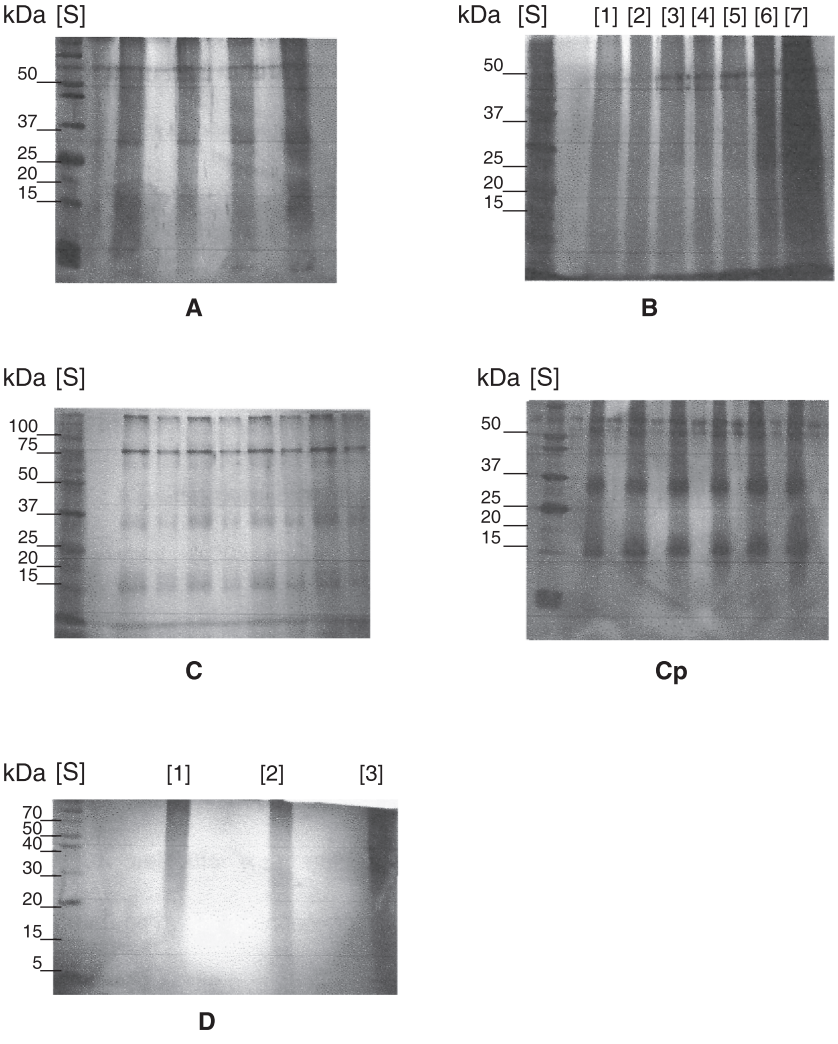


**Figure 2**
SDS-PAGE electrophoresis 12.5%. [S] Protein pattern (Plus Protein™ Dual Xtra Standards, Bio-Rad). [A] *D. pteronyssinus* sample; [B] *B. tropicalis* sample: [1-2] 1:100 dilution, [3-4] 1:50 dilution, [5-6] 1:10 dilution and [7] concentrated precipitated sample; [C] *D. farinae* sample before precipitation and [Cp] after precipitation with acetone; [D] Mite extracts in natura, [1] *B. tropicalis*, [2] *D. farinae* and [3] *D. pteronyssinus*

**Table 2**
Major bands found in 12.5% polyacrylamide gels for *B. tropicalis* (results from three independent experiments)

| Bands | Molecular weight (kDa) | | | $\bar{x}$ [a] | δ [b] | $S_x$ [c] |
|---|---|---|---|---|---|---|
| P1 | 101.0 | NV | NV | 101.0 | – | – |
| P2 | 80.00 | NV | 80.00 | 80.00 | – | – |
| P3 | NV | 35.40 | 36.38 | 35.89 | 0.69 | 0.59 |
| P4 | NV | NV | NV | NV | – | – |
| P5 | NV | NV | NV | NV | – | – |
| P6 | NV | NV | NV | NV | – | – |

[a] Mean, [b] standard deviation, [c] standard error, NV = not viewed, P = protein labeling position on the gel from above.

**Table 3**
Molecular weight of proteins on SDS-PAGE gel 12.5% for *D. farinae* (results from five independent experiments)

| Bands | Molecular weight (kDa) | | | | | $\bar{x}$ [a] | δ [b] | $S_x$ [c] |
|---|---|---|---|---|---|---|---|---|
| P1 | 109.62 | 103.33 | NV | 101.50 | 104.0 | 103.66 | 3.50 | 0.93 |
| P2 | 74.71 | 69.00 | NV | 78.50 | 78.50 | 76.60 | 4.48 | 1.05 |
| P3 | 48.66 | 44.93 | NV | NV | NV | 46.80 | 2.64 | 1.14 |
| P4 | 29.73 | 28.49 | 31.29 | 29.95 | 29.75 | 29.75 | 0.99 | 0.44 |
| P5 | 13.72 | NV | 14.93 | NV | NV | 14.33 | 0.85 | 0.65 |
| P6 | 11.44 | 11.94 | 12.22 | 12.28 | 12.46 | 12.22 | 0.39 | 0.28 |

[a] Mean, [b] standard deviation, [c] standard error, NV = not viewed, P = protein labeling position on the gel from above.

**Table 4**
Molecular weight of proteins on SDS-PAGE gel 12.5% for *D. pteronyssinus* (results from five independent experiments)

| Bands | Molecular weight (kDa) | | | | | $\bar{x}$ [a] | δ [b] | $S_x$ [c] |
|---|---|---|---|---|---|---|---|---|
| P1 | 75.00 | 75.66 | 77.00 | 75.75 | 75.5 | 75.66 | 0.73 | 0.38 |
| P2 | 59.00 | 62.50 | 57.66 | 58.5 | 57.00 | 58.50 | 2.13 | 0.65 |
| P3 | 30.30 | 27.71 | 27.60 | NV | NV | 27.71 | 1.52 | 0.71 |
| P4 | NV | NV | NV | 24.70 | 24.64 | 24.64 | 0.04 | 0.13 |
| P5 | NV | 17.01 | 17.01 | 17.84 | NV | 17.43 | 0.58 | 0.54 |
| P6 | NV | 12.51 | 12.51 | 12.98 | 12.22 | 12.51 | 0.38 | 0.35 |

[a] Mean, [b] standard deviation, [c] standard error, NV = not viewed, P = protein labeling position on the gel from above.

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