

Use of enzyme-developed immunoblots scanned by densitometry to compare lot-to-lot variability of Russian-thistle extracts

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Two different lots of Russian-thistle extracts from five commercial sources were evaluated by isoelectric focusing, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblots of Immobilon transfers from these separations. The immunoblots were developed by an enzyme-linked method and scanned with a densitometer. The two lots of a standardized extracts produced scans that suggested uniformity in allergenic components. Several of the nonstandardized extract lots also appeared quite comparable. With further refinement, immunoblotting with densitometric analysis appears to offer an attractive way to standardize allergen extracts. (J ALLERGY CLIN IMMUNOL 1989;84:529-37.)

The rationale for standardization of allergen extracts has been generally accepted by physicians, scientists, and regulatory bodies concerned with the care of allergic patients. Standardized extracts offer the opportunity to avoid additional needless variability in allergy diagnosis and in allergy immunotherapy.¹⁻³

During the past two decades, an intense effort has been made to develop methods for extract standardization. In 1971, Norman and Lichtenstein⁴ stated that any successful method for extract standardization would have to be more economical and less burdensome than biologic standardization if a full array of standardized extracts was to be developed. An excellent strategy for biologic standardization has been established but requires high technical proficiency in skin testing and is uncomfortable to the human volunteers.⁵

Abbreviations used

AU:	Allergy units
SDS-PAGE:	Sodium dodecyl sulfate-polyacrylamide gel
MW:	Molecular weight
NBT:	Nitro blue tetrazolium
DMF:	<i>N,N</i> -dimethylformamide
IEF:	Isoelectric focusing
pI:	Isoelectric point

A number of *in vitro* techniques have been developed and used to standardize extracts. These include RAST inhibition, crossed immunoelectrophoresis, crossed radioimmuno-electrophoresis, and direct biochemical measurement of major allergenic components of extracts.⁶⁻¹⁰

Recently, there have been many improvements in gel-separation techniques of protein mixtures, both in the gel materials and in the equipment needed to perform these techniques. Concurrently, methods for transferring these separated proteins from gels to fixed matrices have been developed, as well as superior materials to use as the matrices.

These fixed, separated proteins can be overlaid by allergic human serum, and the relevant allergenic proteins can be bound by human IgE-specific antibodies that recognize them. Subsequently, the presence of the human IgE antibodies is detected by labeled antihuman IgE. Most often, this label has been a radioactive isotope. Autoradiography detects the al-

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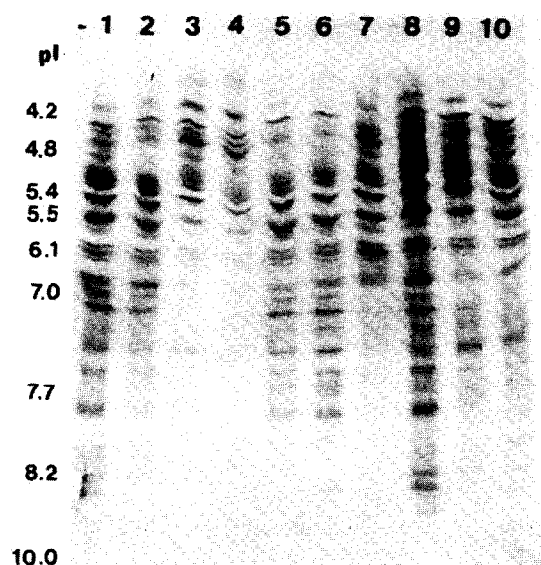


FIG. 1. IEF gel of two different lots of Russian-thistle extracts from five commercial sources. 1 and 2, Berkeley Biologicals; 3 and 4, Greer Laboratories; 5 and 6, 3M Diagnostic Systems; 7 and 8, Hollister Stier; 9 and 10, Center Laboratories.

lergenic protein bands, and the process is called Western blotting or immunoblotting.^{11, 12}

Since radiolabels have inherent difficulties, such as short half-lives of the label and the need for special handling, investigators have adapted enzyme color-development methods to visualize the separated protein bands. In this procedure, the enzyme-developed color reaction occurs at the sites where the allergen, specific human IgE antibody, and enzyme-linked antihuman IgE antibody complexes are present on the matrix. Selected substrate reagents are activated by the enzyme, and a variety of colors can be developed, depending on the enzyme and the substrate chosen.¹⁰

The prints or blots can be carefully scanned by a reflectance densitometer with reproducible patterns. The prints are themselves very stable, and the scan patterns are permanent and can be stored and analyzed by a computer. The patterns can become permanent records of the allergenic components of the extract. The approach is not prohibitively expensive in manpower or reagent costs and has the potential to measure the allergen contents of extracts in both a qualitative and quantitative fashion.

In this article, we will describe our initial experiences in using this method to compare the variations between lots of Russian-thistle extract obtained from five commercial manufacturers. One set of lots was received standardized by biologic methods and by RAST inhibition; the other four sets were not standardized extracts.

MATERIAL AND METHODS

Allergens

Two lots of Russian-thistle extracts were obtained each from Berkeley Biologicals (Berkeley, Calif.), Greer Laboratories (Lenoir, N.C.), 3M Diagnostic Systems (Santa Clara, Calif.), Hollister-Stier Laboratories (Spokane, Wash.), and Center Laboratories (Port Washington, N.Y.). Nonstandardized extracts were preserved in 50% glycerin (Berkeley Biologicals and Hollister-Stier Laboratories), or phenol saline (Greer and Center Laboratories). These nonstandardized extracts were 1/20 wt/vol. The standardized extract (3M Diagnostic Systems) was freeze-dried with separate diluent. The extracts from 3M Diagnostic Systems were reconstituted to 100,000 AU/ml and then treated in the same fashion as the other aqueous extracts.

All extracts were dialyzed against water for 48 hours in dialysis tubing with a pore limit of 3500 MW. The samples were then lyophilized. For some experiments, the dialyzed extract from Berkeley Biologicals was exposed to 0.4% phenol in water for 24 hours, dialyzed again in water, and then lyophilized. The extracts were solubilized in water before electrophoresis.

Serum

Sera were obtained from Russian thistle-allergic patients, who had not had allergy immunotherapy. Each subject had a wheal reaction to Russian thistle-extract (50,000 AU/ml; 3M Diagnostic Systems) skin testing that was at least 5 mm by 5 mm or larger than the wheal response to a glycerol saline control. The sera from 20 patients was pooled and used for allergen recognition in the immunoblot technique.

Electrophoresis and immunoblotting

SDS-PAGE was performed in 12% acrylamide gels at pH 8.3 with a 3.5% polyacrylamide stacking gel. Standard low MW markers (Bio-Rad Laboratories, Richmond, Calif.) were used as migration references. IEF was performed in isogel agarose (FMC Corp., Rockland, Me.) with a continuous pH gradient from 3.0 to 10.0. Standard markers (FMC Corp.) were used for pI determinations. The proteins were developed by staining with Coomassie brilliant blue R 250.

Immunoblotting method

Electrophoresed proteins were transferred to Immobilon paper (Millipore Corp., Bedford, Mass.) in 0.7% acetic acid for IEF gels and 25 mmol/L of Tris-192 mmol/L glycine, pH 8.3, for SDS-PAGE. Unreacted sites on the Immobilon sheets were blocked with 3% human serum albumin (wt/vol) in 0.05 mol/L of Tris HCL buffer, pH 7.4, containing 0.05% Tween 20 (wt/vol). The sheets with the transferred, separated proteins were incubated for 2 hours with a pool of the 20 sera from Russian thistle-allergic patients, with mild agitation. Sheets were thoroughly washed with distilled water. The sheets were incubated for 2 hours with mouse monoclonal antihuman IgE conjugated to alkaline phosphatase (3M Diagnostic Systems). Control reactions

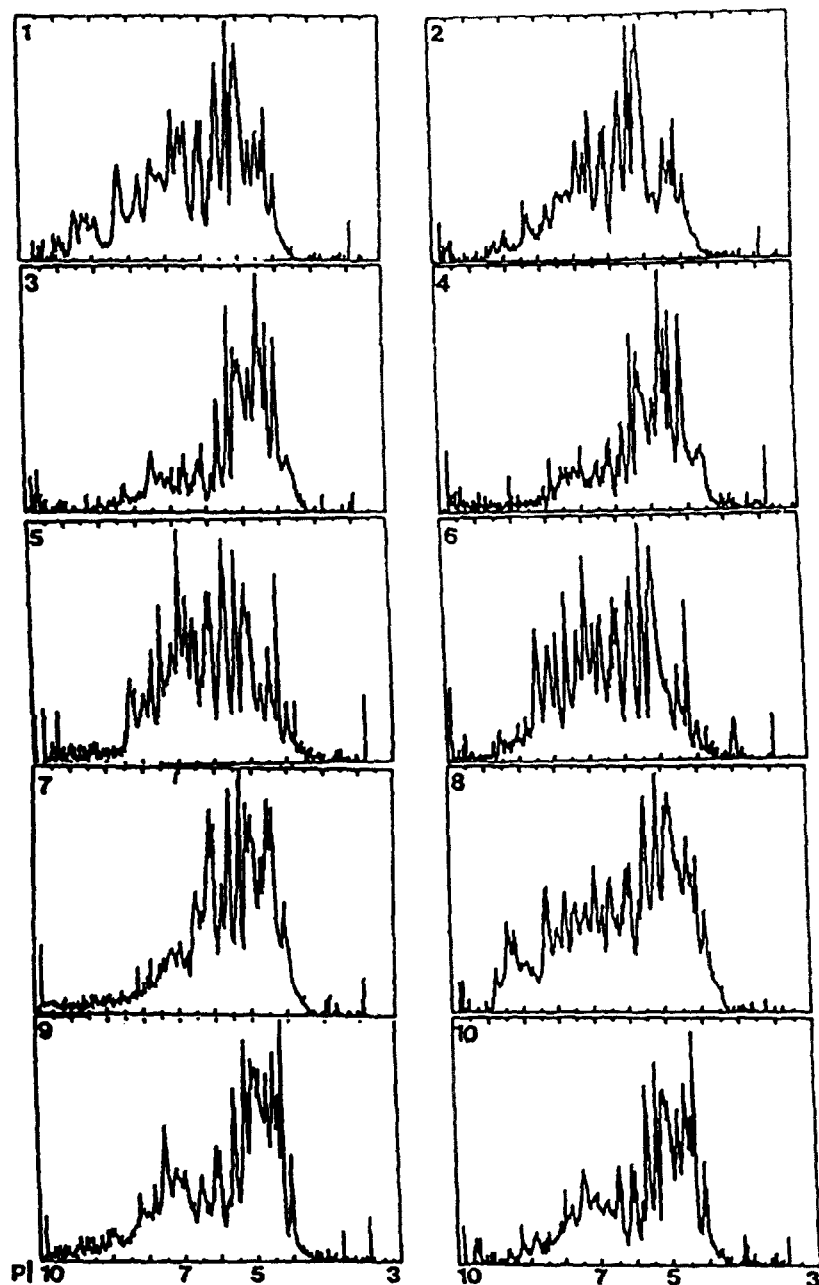


FIG. 2. Densitometry of the isoelectric focused extracts illustrated in Fig. 1. Same numbering applies.

were performed with cord blood serum. Specific bands were developed with the substrate NBT and 5-bromo-4-chloro-3-indolyl phosphate (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) following the instruction manual for alkaline phosphatase conjugates obtained from Bio-Rad Chemical Division. A 70% DMF solution was prepared by mixing 0.7 ml of DMF with 0.3 ml of H₂O; 30 mg of NBT was then dissolved in this solution. A second solution was prepared by dissolving 15 mg of 5-bromo-4-chloro-3-indolyl phosphate in 1 ml of DMF. The two solutions were then mixed with 100 ml of carbonate buffer (0.1 mol/L of

NaHCO₃ and 1.0 mmol/L of MgCl₂, pH 9.8) just before starting color development. The NBT substrate was used primarily because of stability of the color reaction.

Densitometry

The stained bands for the Russian-thistle extracts that were developed in the gel or on Immobilon were scanned for absorption (gels) or reflectance (immunoblots) with a Hoefer GS 300 scanning densitometer (Hoefer Scientific Instruments, San Francisco, Calif.) attached on-line to an IBM XT computer. Analysis was performed with the GS

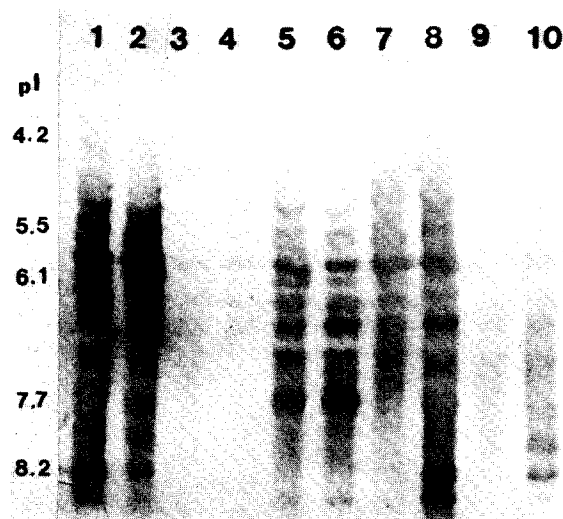


FIG. 3. Immunoblot of isoelectric-focused Russian-thistle extracts. The bands were developed with pooled sera from 20 sensitive subjects. The number remains the same.

250 data system. Quantitation of the scanned bands was by integration of the peak areas. However, careful review of the immunoblots and scans revealed the peaks included more than one protein band. The light source was not discrete enough to discriminate the bands that were close together. Therefore, comparisons of the scans were made on a visual basis with overlays rather than on a quantitative basis.

RESULTS

IEF of extracts

The two lots of Russian-thistle extracts from the five different companies were compared by IEF. Preliminary experiments were necessary to determine the optimum concentrations of extract required for adequate protein band development because we discovered important differences for each company. The optimum band development occurred when we used 5 μ l aliquots containing either 20 mg/ml of extract (Berkeley Biologicals and 3M Diagnostic Systems), 30 mg/ml of extract (Greer and Hollister-Stier Laboratories), or 50 mg/ml (Center Laboratories) (Fig. 1). At these concentrations, most protein bands focused between pIs of 4.0 to 10.0 for all the extracts.

Densitometry of the isoelectric-focused extracts (Fig. 2) demonstrated that the lot to reproducibility was reasonably good for each supplier. The visualized differences between the lots illustrated in Fig. 1 were also detected by densitometry. The most notable between-lot differences were in the range of pI 7.0 and 10.0. Proteins within this pI range made up as much as 30% of the total protein content from several supplies (Berkeley Biologicals and 3M Diagnostic

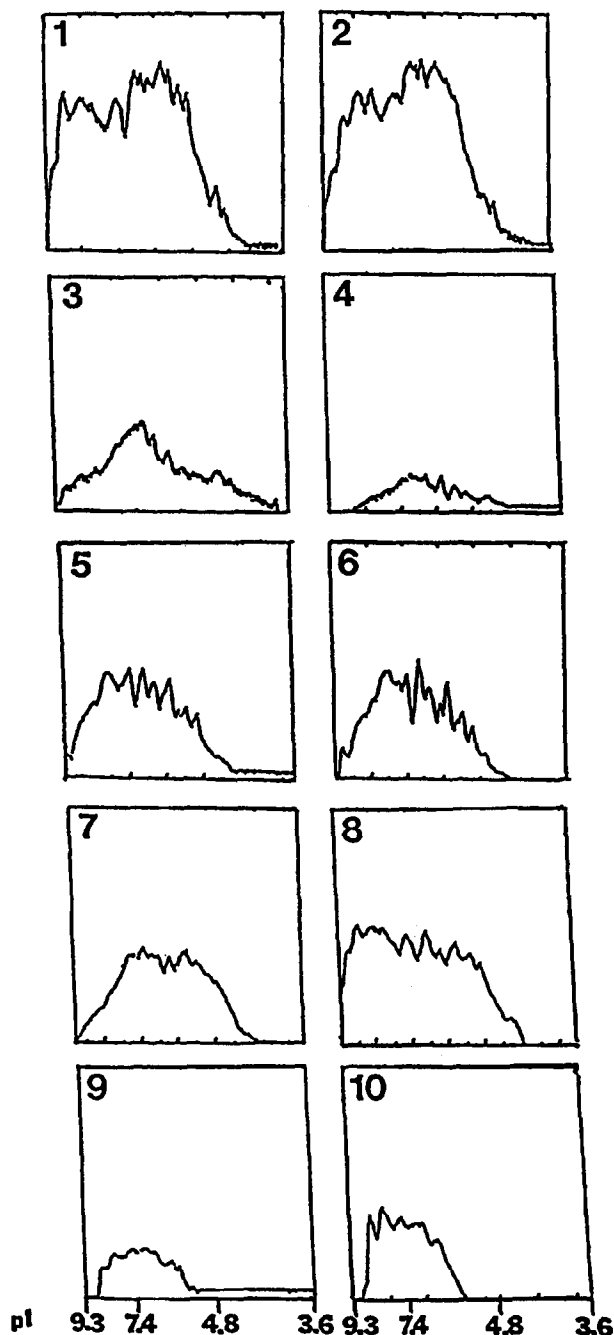


FIG. 4. Densitometry of the immunoblot of the isoelectric-focused Russian-thistle extracts illustrated in Fig. 3.

Systems) but were completely absent in extracts of one supplier (Greer Laboratories).

The results of the immunoreaction of the pooled sera from allergic patients with the blot of the isoelectric-focused extracts are illustrated in Fig. 3. Comparison of the recognized bands of the immunoblot with the isoelectric-focused gel in Fig. 1 re-

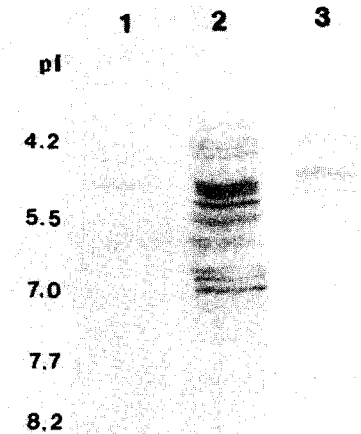


FIG. 5. Immunoblot from an isoelectric-focused gel of Russian thistle extract from Berkeley Biologicals. *Lane 1*, 20 µg/ml of pheno-treated extract; *lane 2*, 20 µg/ml of untreated extract; *lane 3*, 100 µg/ml of phenol-treated extract. See discussion in text.

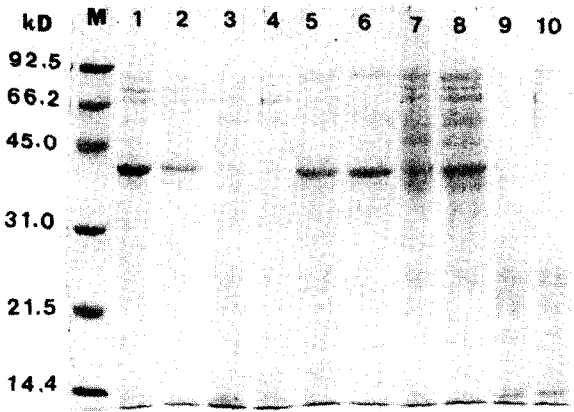


FIG. 6. SDS-PAGE of two different lots of Russian-thistle extracts from five commercial sources. *1 and 2*, Berkeley Biologicals; *3 and 4*, Greer Laboratories; *5 and 6*, 3M Diagnostic Systems; *7 and 8*, Hollister-Stier Laboratories; *9 and 10*, Center Laboratories.

vealed that most of the separated proteins reacted with human IgE antibodies except for the extracts from two of the suppliers (Greer and Center Laboratories). Densitometry of the immunoblots provided patterns of recognition by the sera from allergic patients (Fig. 4).

The immunoblot of the extracts that did not demonstrate band recognition by the sera from allergic patients also did not stain with Coomassie brilliant blue or amido black. This finding suggested that the proteins from the gel had not transferred adequately.

The effect of the phenol preservative used in the two nontransferring extracts was measured by pre-treating extract that had successfully transferred pre-

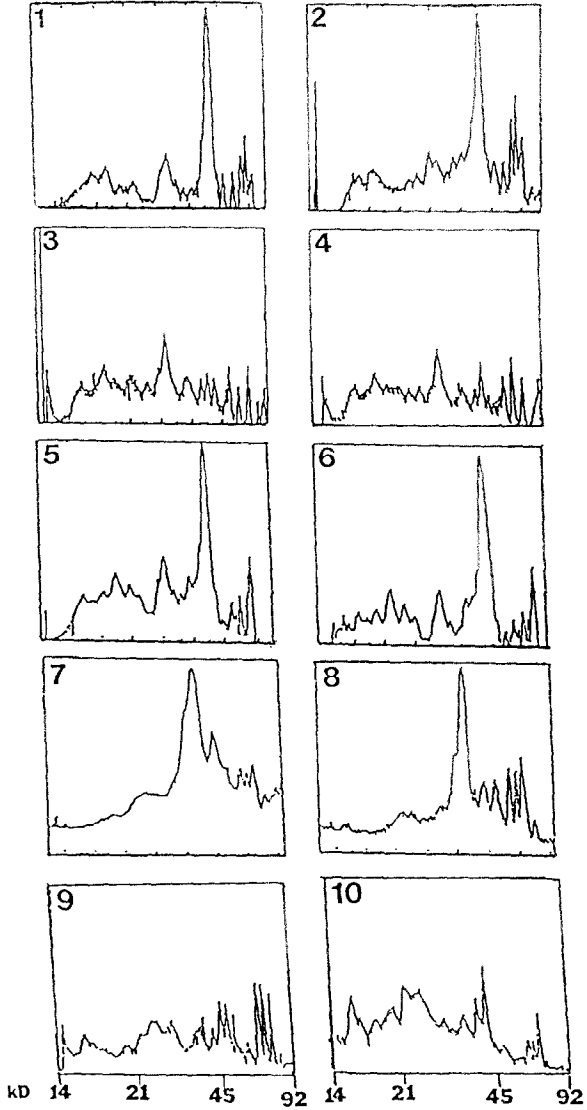


FIG. 7. Densitometry of the Russian-thistle extracts from the SDS-PAGE illustrated in Fig. 6.

viously with 0.4% phenol. Dialyzed extract from Berkeley Biologicals was exposed to phenol for 24 hours, redialyzed, and lyophilized. This material was isoelectric focused on gel, transferred to Immobilon, and immunoblotted. Many of the proteins found on the immunoblot of the original material could not be found after phenol treatment. A higher concentration of extract (100 µg/ml) had to be applied to provide enough material for stainable bands to be visualized on the gel.

Patterns found on the enzyme-developed immunoblots and in blots stained for proteins with Coomassie brilliant blue or amido black were similar to that of the gels. This finding indicated that the phenol treatment did not interfere with electrophoretic trans-

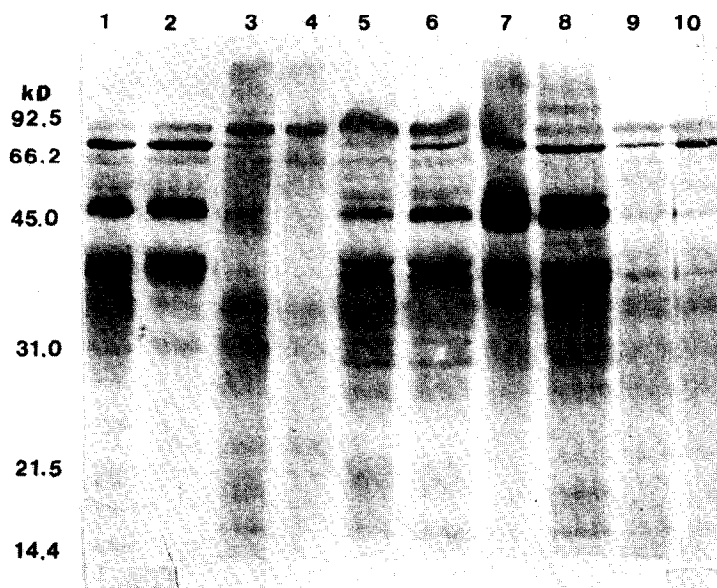


FIG. 8. Immunoblot of the Russian-thistle extracts from the SDS-PAGE. The bands were developed with pooled sera from 20 sensitive subjects. 1 and 2, Berkeley Biologicals; 3 and 4, Greer Laboratories; 5 and 6, 3M Diagnostic Systems; 7 and 8, Hollister-Stier Laboratories; 9 and 10, Center Laboratories.

fer of proteins from the gel to the Immobilon membrane (Fig. 5). The extract was degraded during the treatment and was lost before electrophoretic transfer. Higher concentrations of extract were required for band development in both the gel and the Immobilon membrane.

SDS-PAGE

It was again necessary to adjust the concentration of the extracts required for optimum band development in SDS-PAGE gels; 10 μ l aliquots of 2.5 mg/ml (Berkeley Biologicals, 3M Diagnostic Systems, and Hollister-Stier Laboratories), 5.0 mg/ml (Greer Laboratories), and 10 mg/ml (Center Laboratories) were used.

The SDS-PAGE patterns of the extracts were similar from lot to lot. There were marked differences between the freeze-dried, the glycerosaline-preserved, and the phenol-preserved extracts (Fig. 6). One protein of 40,000 daltons was prominent in the extracts from Berkeley Biologicals, 3M Diagnostic Systems, and Hollister-Stier Laboratories. It was only a minor component in extracts from Greer and Center Laboratories. Another prominent band of MW 92,000 was present in the extracts from Hollister-Stier Laboratories but absent in all the other extracts except for one lot from Berkeley Biologicals (*lane 2*). Visual differences in band concentrations, even with the application adjustments, were striking.

Densitometry of the SDS-PAGE (Fig. 7) illustrates these density differences.

Immunoblot from the SDS-PAGE revealed that most of the proteins reacted with the pooled sera (Fig. 8). Three prominent bands of 40,000, 50,000, and 75,000 daltons were present in varying amounts in all extracts. The extracts from Greer and Hollister-Stier Laboratories had immunoreactive bands of MW >90,000 that were not present in the other extracts. The electrophoretic transfer of the proteins to Immobilon was not a problem from SDS-PAGE gels.

The densitometric patterns demonstrated the comparability of the two lots of standardized extracts. The patterns of the two glycerinated extracts also were very similar (Fig. 9).

DISCUSSION

Our original goal was to study immunoblot analysis with densitometric measurement as a means to evaluate and ultimately to standardize allergen extracts. Using this technique, we compared the variability between two lots of the same extract from different manufacturers, as well as differences between the extracts, as provided for clinical use. We were particularly interested in comparing the nonstandardized extracts to the standardized extracts.

Incidentally, because of need, we also evaluated the effects of phenol preservative in allergy extracts.

Our first objective was achieved. The combina-

tion of immunoblotting and scanning densitometry worked. The densitometric patterns of the immunoblots were crisp. Visual comparisons were easily made, and reactive protein (allergen and human IgE antibody) differences present on the immunoblot were observed in the scan patterns. Variations between lots from the same manufacturer and among lots from different manufacturers were easy to detect.

The patterns were stored in a computer, and we have begun to appreciate the detailed analysis possible. In this present study, we found that limiting factors were the discriminatory ability of our scanning densitometer and the homogeneity of the separation gels.

Since this work was performed, commercially available minigels of higher uniformity have become available. Laser-light sources for densitometry have become less expensive. The programs available for analysis of the scan patterns are more sophisticated.

It is our considered opinion that this combination of immunoblotting, laser densitometric scanning, and computer analysis will make it possible to address the problem of extract standardization as defined by Norman and Lichtenstein.⁴ Work in this direction is underway in our laboratory.

The technique can measure the presence or absence of, and the relative amount of, allergenic components based on human antibody recognition. In future studies, comparisons to the presently recognized methods appear indicated and relationships appear established.

A significant advantage of this technique is computer pattern storage and analysis. After an agreed-on, standard allergen extract is achieved for the allergen in question, by the usual means, its pattern of reaction with a pool of sera from allergic patients can be measured and stored in the computer. Future extracts of this allergen that are manufactured can be reacted with this sera pool, and the pattern of the immunoblot can be compared to the pattern of the standard. This is a relatively inexpensive and rapid method of comparison. The standard allergen extract will be preserved, since it must only be used when a new pool of sera from allergic patients is needed to demonstrate a new standard pattern. It is likely with a pool of sera from a large number of allergic subjects that the new pattern may be similar to the pattern established as precedent. Acceptable variations in the manufactured lots for clinical use can be determined by future study. Completion of the above steps will make standardization of each new lot less costly to the manufacturers. It is our opinion that the reduction in costs will make it economically viable to standardize all allergen extracts, including special regional

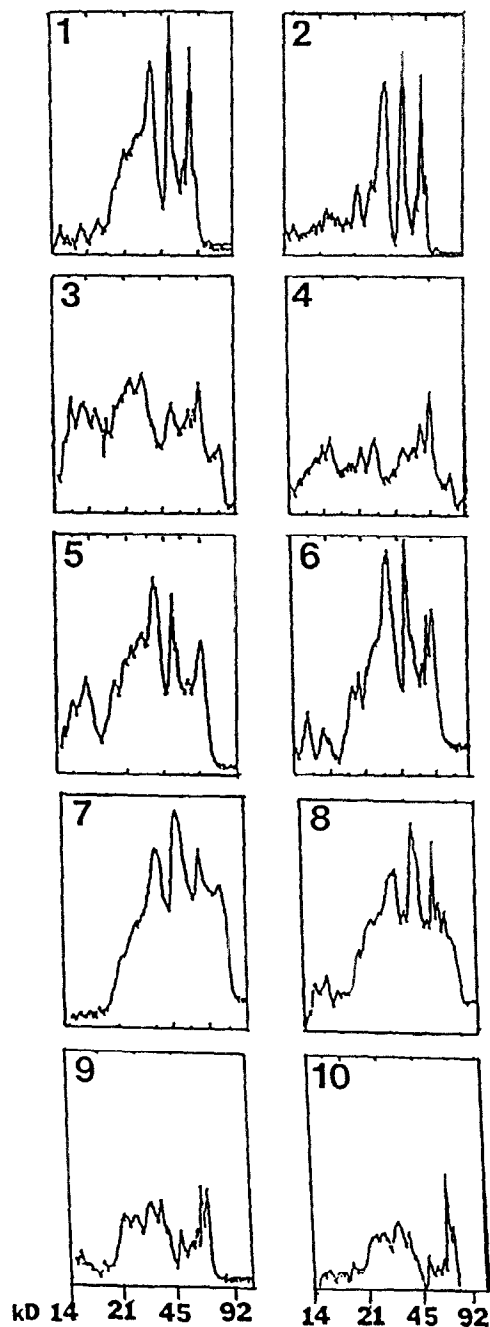


Fig. 9. Densitometry of the immunoblot of the Russian-thistle extracts after SDS-PAGE illustrated in Fig. 8.

allergens. After the initial effort and costs are met of the use of the presently recognized techniques to obtain a standard, subsequent expenses would be minimal and justify continued manufacture.

To accomplish this goal, a major project will be the collection of standard sera pools from allergic patients for each of the allergen extracts. It is probable that the same pool can serve to form a pattern with more

than one extract. For some allergens, single sera pools may be necessary. As mentioned above, a suitable extract will require all allergen bands with a certain percentage distribution of these bands.

With sophisticated computer analysis, it may even become possible to predict the results of immunoblotting from the gel separations. If such refinement is possible and precise enough, the costs involved in standardization would be further reduced and bring us closer to the stated goal of affordable better quality materials for allergy diagnosis and therapy.

In this study, the standardized extracts did demonstrate very good comparability by the techniques used. Several other extracts also were quite comparable from lot to lot. We did not have an opportunity to examine multiple different lots from any of the manufacturers. It is likely that comparability between lots of standardized extract would continue. Such expectation of nonstandardized extracts appears less reasonable.

The deleterious effects of phenol as a preservative for allergen extracts have been addressed by a number of previous investigators.¹³⁻¹⁵ As revealed by the absence of allergen bands, both on the gels, the transfers, and the immunoblots, even with concentration adjustments, the phenol saline preservative is far from ideal. The simple study performed on the 24-hour effects of phenol suggests that use of the proper preservative is an important problem in allergen standardization for clinical allergy.

We chose to make immunoblots of transfers from SDS-PAGE, although we recognized that the proteins are denatured in the process. Clearly, this may impact on the epitopes available for recognition by human antibodies. However, we believe the recognition patterns found in this study are valid. They provide insights into the extract components not achievable with IEF, MWs of the protein antigens being the most obvious example. Other investigators have used this approach to extract evaluation.¹⁶

Allergen analysis by immunoblotting has been reported for foods, pollen, molds, and animal proteins.¹⁶⁻¹⁹ In most cases, autoradiography was used to detect the antibody bound to the proteins. However, the enzyme color-label technique has been also used.¹⁷

The concept of quantitating the immunoprints is based on the success achieved in immunodot techniques, which are generally used with unseparated allergen mixtures. The intensity of the color reaction is quantitated visually or by a densitometer.

In summary, analysis of lot-to-lot variability of Russian-thistle extracts by immunoblotting and scanning densitometry revealed good comparability of

standardized extracts and several nonstandardized extracts. Extracts preserved in phenol saline appeared to have less allergenic protein content than the freeze-dried standardized extract or the glycerosaline extracts. The combination of gel separation, immunoblotting, and scanning densitometry, along with computer analysis, offers attractive possibilities to evaluate allergens.

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