

Production and testing of an international reference standard of short ragweed pollen extract

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A lyophilized candidate International Reference Standard of short ragweed pollen extract was prepared by use of defined source material. In preliminary experiments, this extract was demonstrated by RAST inhibition and crossed radioimmuno-electrophoresis assays to contain several well-characterized ragweed allergens and to contain multiple antigenic bands by crossed immunoelectrophoresis analysis. In a subsequent multinational collaborative study involving 12 laboratories in five countries, the candidate extract was compared with existing national reference or commercial ragweed extracts by a variety of immunochemical, biochemical, and physicochemical procedures. The candidate extract could be used to assign relative orders of potency to the comparison-test extracts. In separate studies, the candidate extract was demonstrated to be stable when it was stored at either -20°C or $+5^{\circ}\text{C}$ for at least 2 yr. The candidate extract has been accepted as an International Reference Standard with an assigned arbitrary potency of 100,000 units per ampule. (J ALLERGY CLIN IMMUNOL 73:790-800, 1984.)

In 1981 the Allergen Standardization Subcommittee of the International Union of Immunologic Societies undertook the production of several allergen extracts that would meet WHO specifications as IRPs.¹ The aims of the project were to: (1) establish the suitability of a selected preparation to serve as the IRP, which once established could be used by any regulatory authority, manufacturer, or research group, (2) demonstrate that potency assays, such as RAST inhibition, quantitative immunoelectrophore-

Abbreviations used

WHO:	World Health Organization
IRP:	International Reference Preparation
SRW:	Short ragweed
FDA:	Food and Drug Administration
Ag:	Antigen
BSA:	Bovine serum albumin
CIE:	Crossed immunoelectrophoresis
CRIE:	Crossed radioimmuno-electrophoresis
ELISA:	Enzyme-linked immunosorbent assay
HPLC:	High-pressure liquid chromatography
TLIEF:	Thin layer isoelectrofocusing
HSA:	Human serum albumin

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sis, and leukocyte histamine release could be used with the IRP to assign a relative potency to extracts of unknown potency, and (3) collect data that would enable a sensible order of magnitude to be assigned to an arbitrary unitage.

SRW pollen extract was selected to be one of the initial allergens for the development of an IRP. Because of the WHO requirement that IRP be packaged in glass ampules,¹ the commercial SRW allergen extracts produced by United States manufacturers failed

to meet the established criteria for IRP materials. A working group consisting of Drs. J. W. Yunginger (coordinator), H. Baer, R. A. Goldstein, P. S. Norman, and D. A. Levy undertook the responsibility of producing a candidate IRP of SRW pollen extract.

Preliminary investigations were undertaken by Drs. H. Baer, H. Lowenstein, and J. W. Yunginger to determine the suitability and stability of the candidate IRP SRW pollen extract. This candidate IRP SRW pollen extract was then compared to several other SRW allergen extracts in an international collaborative study designed to confirm the usefulness of the former extract as an international standard allergen extract preparation. The results of these investigations are the subject of this article.

PRELIMINARY INVESTIGATIONS

Material and methods

Candidate SRW pollen extract

Equal parts of SRW, *Ambrosia elatior*, from each of four different years of collection (1962, 1964, 1978, and 1979) were donated by Dr. H. Baer, Office of Biologics, United States FDA. These pollen lots had been collected by Greer Laboratories, Lenoir, N. C., then shipped to Hollister-Stier Laboratories, Spokane, Wash. They were dried under vacuum, sealed in lined, metal-topped glass jars, flushed with nitrogen, sealed with beeswax, and stored at 2 to 10° C. Each pollen lot was examined by scanning electron microscopy for foreign debris and plant parts and to verify the identity of the pollen. The pollen grains were found to be compatible with those identified as short ragweed, and the degree of foreign debris and plant parts was found to be within the acceptable limits formulated by the investigative subgroup and accepted by the Allergen Standardization Subcommittee (Steering Committee minutes, Geneva, 1981).

The individual SRW pollen lots were pooled, defatted with petroleum ether in a Soxhlet apparatus for 6 hr, and dried. The extraction (100 gm defatted SRW pollen in 1000 ml deionized water) was carried out at room temperature for 22 hr with gentle stirring. The SRW extract was isolated by centrifugation, passed sequentially through 0.45 μ m and 0.22 μ m filters, then freeze-dried immediately in 0.3 ml aliquots by the American Type Culture Collection, Rockville, Md., in scored glass ampules, and sealed under nitrogen.

Ragweed RAST reagent

A solid-phase SRW reagent for the subsequent collaborative study was prepared by extracting a separate lot of undefatted SRW pollen in water and partially purifying the extract over a calibrated Sephadex G-25 column.² This extract was subsequently demonstrated to contain the purified ragweed allergens AgE, AgK, Ra3, Ra4, and Ra5 by RAST inhibition studies.³ The partially purified SRW extract (206 mg dry weight) was reacted with Matrex Pel 102 acrylic beads (0.5 gm, Amicon Corp., Lexington, Mass.) as 4° C overnight,⁴ and the coupled SRW (polymer no. 1) was isolated by centrifugation. The supernatant fluid was reacted in

identical fashion with a fresh 0.5 gm aliquot of Matrex to produce SRW polymer no. 2, and the process was repeated until eight SRW polymers were obtained. Each polymer was washed 9 to 16 times with 30 to 40 ml aliquots of borate buffer (0.2M H₃BO₃-0.04M NaOH-0.16M NaCl, pH 8.0) until the 277 nm absorbance was ≤ 0.060 and then suspended at 1 mg/ml concentration in RAST diluent (0.1M phosphate buffer containing 0.2% BSA, 1% Tween 20, and 0.1% sodium azide). Each SRW polymer was tested with varying quantities (0.2 to 50 μ l) of a serum pool derived from 12 SRW-sensitive patients who had not previously received immunotherapy. The dose-response curves produced with polymers nos. 1 through 5 were quite similar, whereas the slope of polymer no. 6 curve was less steep; the curves produced by polymers nos. 7 and 8 were relatively flat. We concluded that all relevant allergens were coupled to the Matrex during the first six passages, and accordingly polymers nos. 1 through 6 were pooled to produce 3 L of SRW-Matrex at 1 mg/ml in RAST diluent.

IgE-antibody pool

We collected an IgE-antibody pool containing equal parts of defibrinated delipidated plasma from 10 persons with typical clinical histories of ragweed hay fever who had not previously received SRW immunotherapy. The IgE-antibody contents of pilot sera from each donor were measured by RAST by use of SRW-coupled discs from Pharmacia Diagnostics, Piscataway, N. J., as well as by mini-RAST⁵ by use of AgE, AgK, Ra3, and Ra5-microcrystalline cellulose particles prepared in our laboratory.³ All donors scored as class 4 with the Pharmacia grading system. Plasma donors were in good general health, and each plasma was free of hepatitis B surface antigen. After defibrination by dialysis against Ca⁺⁺ containing buffers, excess Ca⁺⁺ was removed by dialysis against 0.15M NaCl and the plasmas were then delipidated by Freon-113 treatment⁶ by use of a ratio of two parts plasma to one part Freon.

Immunoelectrophoretic analysis

The immunoelectrophoresis methods were generally performed as previously described;⁷ gels were stained with Coomassie brilliant blue. CIE studies were undertaken to study the number and relative quantity of antigenic determinants in the candidate SRW extract by use of rabbit anti-*Ambrosia elatior* antibody (Allergologisk Laboratorium, ALK, Copenhagen, Denmark). A 1.5-millimeter thick agarose gel (1% w/v) was poured on a 7 by 7 cm glass plate with the application hole centrally located. The candidate SRW extract was reconstituted in 0.15 ml of H₂O; 5 μ l of the extract was added to the well, and the first dimension electrophoresis was carried out at 10 v/cm for 30 min. Surplus gel was cut away and replaced by a 1.3-millimeter thick agarose gel containing 10 μ l of antibody per square centimeter and electrophoresed at 1.5 v/cm overnight. The results of two CIE plates run at an 8-week interval by use of different ampules of the candidate SRW pollen extract indicated that the major allergens were present when results were compared with a published line drawing of another SRW pollen extract.⁸

Allergenicity of some of the SRW extract components

TABLE I. Inhibitory activity of the candidate and companion SRW extracts in the crude SRW RAST and in three purified SRW allergen RASTS

Inhibitor	Dilution	SRW-Matrex RAST		AgE-Cellulose RAST		Ra3-Cellulose RAST		Ra5-Cellulose RAST	
		Counts bound (%)	Inhibition (%)	Counts bound (%)	Inhibition (%)	Counts bound (%)	Inhibition (%)	Counts bound (%)	Inhibition (%)
None	—	14.38	—	11.92	—	7.45	—	6.42	—
Candidate SRW extract	Neat	0.25	98	0.41	97	0.67	91	0.35	95
	1 to 10	0.43	97	0.75	94	1.55	79	0.59	91
	1 to 100	1.61	89	2.16	82	4.19	44	1.48	77
	1 to 1000	6.03	58	6.09	49	5.04	32	4.16	35
United States FDA SRW reference extract	Neat	0.40	97	0.52	96	0.72	90	0.42	93
	1 to 10	0.91	94	1.38	88	1.67	78	0.58	91
	1 to 100	3.07	79	3.34	72	3.67	51	1.38	79
	1 to 1000	8.47	41	8.17	31	5.60	25	3.57	44
Partially-purified SRW	5190 µg/ml	0.26	98	0.37	97	0.65	91	0.29	95
	519 µg/ml	0.51	96	1.07	91	2.03	73	0.98	85
	51.9 µg/ml	1.77	88	2.64	78	3.64	51	1.86	71
	5.19 µg/ml	6.24	57	6.28	47	7.33	2	4.76	26

Values listed are the mean of duplicate determinations.

TABLE II. SRW extracts used in the collaborative study

Code letter	Identification	Reconstitution volume (ml)
A	National SRW reference	0.5
B	Candidate SRW extract	1.0
C	Commercial SRW extract	10.0
D	Commercial SRW extract	10.0
E	Same as extract D but diluted 1:5	1.0
F	Coded duplicate of extract B	1.0

was confirmed by CRIE by use of pooled sera from ragweed-allergic patients (pool 0600, Dr. D. G. Marsh). Unstained CIE plates were incubated with 7.5 ml of the serum pool diluted 1 to 15 for 20 hrs. The plates were washed and incubated with 7.5 ml of ¹²⁵I-labeled-anti-IgE (Pharmacia Laboratories) for 20 hr, after which plates were again washed. Autoradiography was performed on x-ray film (Kodak XL-1) for 7 days at -20° C with an intensifier screen. Several of the bands characteristically identified as SRW allergens were identified from the radiostained CRIE plates including Ags 5a, 26, 27, 31 (Ra4), 35, and Ra5.⁹

In addition, the identity of AgE and AgK bands was confirmed by use of crossed-line immunoelectrophoresis with purified preparations of AgE and AgK provided by the courtesy of Dr. T. P. King. The presence of Ra3 in the candidate SRW extract was confirmed by use of mono-specific antibody to Ra3 kindly provided by Dr. D. G. Marsh. Finally, a known quantity of Ra5 was added to the

extract before testing by CIE to intensify and thus identify the resulting Ra5 precipitin band.

TLIEF

The candidate SRW pollen extract was separated by TLIEF by use of 5% polyacrylamide gel (pH 3 to 10) and stained with Coomassie brilliant blue. Companion test extracts included the United States SRW reference extract (lot EI-Ra₅, May 1972), the partially purified SRW extract used to prepare the SRW-Matrex polymer described previously, and an AgE preparation obtained from the Research Resources Branch of the National Institute of Allergy and Infectious Diseases. The pattern of banding produced by each crude SRW extract was quite similar.

RAST-inhibition studies

The allergenicity of the candidate SRW extract was tested by mini-RAST inhibition assay¹⁰ by use of pooled sera from ragweed-allergic individuals and various crude and purified SRW solid-phase allergens. One vial of the lyophilized candidate SRW extract was reconstituted with 1.0 ml RAST diluent. A companion lyophilized test extract, the United States FDA SRW reference preparation, was reconstituted in identical fashion, and additional tenfold dilutions of each extract were prepared in RAST diluent.

The mini-RAST inhibition assay was performed as described previously by use of the SRW-Matrex, AgE-cellulose, Ra3-cellulose, or Ra5-cellulose polymers (50 µl) and the IgE antibody pool (10 to 20 µl) described above. The candidate SRW extract, the United States FDA SRW reference extract, and the partially purified SRW extract (from which the SRW-Matrex polymer was prepared) were added at varying dilutions (50 µl) as first-stage RAST inhibitors; total reaction volume was 250 µl. RAST-inhibition results are listed in Table I. All three test extracts produced

TABLE III. Geometric mean potency ratios ($\pm$ SD) of all test extracts relative to extract B, both for individual laboratories and for all laboratories combined

Laboratory	Potency ratios				
	A	C	D	E	F(=B)
1	1.20 $\pm$ 0.10	0.24 $\pm$ 0.05	0.06 $\pm$ 0.08	0.18 $\pm$ 0.20	1.36 $\pm$ 0.24
2	1.49 $\pm$ 0.17	2.23 $\pm$ 0.29*	0.67 $\pm$ 0.31	0.13 $\pm$ 0.20	1.19 $\pm$ 0.08
3	1.80 $\pm$ 0.02	2.34 $\pm$ 0.26	0.79 $\pm$ 0.39	0.13 $\pm$ 0.07	1.18 $\pm$ 0.16
4	1.55 $\pm$ 0.04	1.63 $\pm$ 0.13	0.68 $\pm$ 0.09	0.19 $\pm$ 0.16	0.89 $\pm$ 0.11
5	1.67 $\pm$ 0.20	1.25 $\pm$ 0.05	0.70 $\pm$ 0.04	0.11 $\pm$ 0.15	0.81 $\pm$ 0.09
6	1.59 $\pm$ 0.30	1.99 $\pm$ 0.35	2.22 $\pm$ 0.11	ND	1.09 $\pm$ 0.22
8	2.80 $\pm$ 0.16	2.35 $\pm$ 0.27	1.67 $\pm$ 0.28	0.70 $\pm$ 0.12	2.04 $\pm$ 0.03
9	1.83 $\pm$ 0.08	1.32 $\pm$ 0.02	0.72 $\pm$ 0.08	0.11 $\pm$ 0.11	0.87 $\pm$ 0.02
10	1.07 $\pm$ 0.10	2.73 $\pm$ 0.12	0.88 $\pm$ 0.08	0.11 $\pm$ 0.08	0.64 $\pm$ 0.16
11	1.66 $\pm$ 0.10	1.54 $\pm$ 0.07	0.59 $\pm$ 0.07	0.11 $\pm$ 0.06	0.91 $\pm$ 0.04
Overall mean (N = 10)	1.62 $\pm$ 0.11	1.52 $\pm$ 0.30	0.68 $\pm$ 0.42	0.16 $\pm$ 0.26†	1.05 $\pm$ 0.14

ND = not done.

*Mean of two assays.

†N = 9.

significant RAST inhibition when the extracts were tested against both crude and purified SRW polymers. The candidate SRW extract contained nearly the same allergenic activity as the 5 mg/ml partially purified SRW extract.

Protein content

The candidate SRW extract was demonstrated to have a protein nitrogen content of 1.728 mg of nitrogen per milliliter as measured by the Kjeldahl method¹¹ and to have a moisture content of 0.90%, determined by the gravimetric method; both nitrogen and moisture content were measured in the laboratory of Dr. Harold Baer.

General sterility

The candidate SRW extract successfully met the United States federal specifications and regulations for general sterility tests as outlined in Section 610.12 of Title 21 of the Code of Federal Regulations.

COLLABORATIVE STUDY

An international collaborative study involving the candidate SRW extract was organized in accordance with published guidelines¹. Twelve laboratories responded to an invitation to participate in this study (Appendix). Laboratories were assigned a code number after agreeing to perform selected testing of the SRW preparations.

Conduct of the study

The lyophilized SRW extracts used for the collaborative study are listed in Table II. These included coded duplicates of the candidate SRW pollen extract as well as SRW extracts from commercial suppliers or regulatory agencies. These companion extracts were chosen in part because they already existed in lyophilized form (obviating the need for freeze-

drying) and in part because they satisfied WHO guidelines for companion test extracts¹.

To provide a test extract whose potency was markedly reduced, we also prepared a 1:5 dilution of one of the commercial SRW extracts in 0.1M ammonium bicarbonate after which 1.0 ml of this dilution was placed in ampules, lyophilized, and sealed under nitrogen.

Each participating laboratory was supplied ampules of the lyophilized candidate SRW extract, ampules of lyophilized companion SRW pollen extracts, ampules of lyophilized individual and pooled sera containing elevated IgE antibodies to SRW, and a supply of the SRW-Matrix polymer described previously for RAST-inhibition studies. Participants were urged to evaluate the SRW extracts by use of both their in-house assay methods and also the "standard" procedures agreed to previously by the Allergen Standardization Subcommittee members. Instruction sheets for performing CIE/CRIE and RAST-inhibition assays were provided to each participating laboratory. The materials were supplied in the fall of 1982, and data were received from December 1982 through March 1983.

Statistical treatment

All laboratories submitted their raw RAST-inhibition data that were analyzed as the log dose (μ l) versus the percent inhibition response. The inhibition curve of each individual SRW extract was analyzed by polynomial regression analysis that included at least three test concentrations to give a correlation coefficient of >0.9 . Parallelism between inhibition

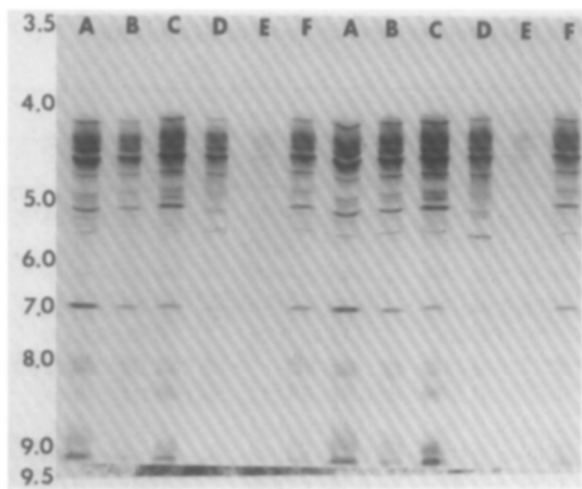


FIG. 1. TLIEF patterns of SRW extracts; pH gradient (3.5 to 9.5) is demonstrated to *left*. Six samples to *left* were single-applied, whereas six samples to *right* were double-applied.

curves for extracts B and F (coded duplicates) and D and E (coded dilution) was tested by one-way analysis of variance, and probability values less than 0.01 were considered to be statistically significant. Comparisons were also made to determine potency ratios relative to the candidate SRW extract (extract B). The ratios were calculated from the log dose response at the 50% inhibition level by use of the slope and y-intercept values provided by the regression analysis program. The geometric means and the standard deviations of the potency ratios from the individual assays from each laboratory were calculated, and an overall geometric mean was calculated to assign a relative potency to each extract.

RAST inhibition

RAST-inhibition data were received from 10 of the 12 laboratories. Eight of the 10 laboratories used the mini-RAST reagents (SRW-Matrex, IgE-antibody pool) and protocol provided with the test samples. The remaining two laboratories (nos. 1 and 3) performed their in-house RAST-inhibition assays by use of SRW antigen coupled to cyanogen bromide-activated paper discs. Three to five independent RAST-inhibition assays were performed by each laboratory.

Nine of the 10 laboratories reconstituted the lyophilized SRW test extracts in the recommended volumes. Laboratory no. 1 used a more concentrated stock solution for each of the test extracts; variable dilutions were then prepared from these stock solutions for use in the assays. The raw data submitted by each laboratory were used to prepare RAST-inhibition curves for each extract in each assay from each laboratory; these were tested by regression analysis for

linearity. In general, the slopes of the RAST-inhibition curves were similar. In nearly all cases, extract E was correctly identified as containing lesser allergenic activity, whereas the allergenic activities of the other five test extracts were usually within tenfold of one another. In general, the RAST-inhibition curves of extracts B and F, the coded duplicates, were quite similar, which indicated that the assay was being done with a good degree of precision in each laboratory.

To generate more quantitative data concerning the relative allergenic potencies of the SRW test extracts, we calculated the volume of each reconstituted extract required to produce 50% inhibition of RAST binding.¹² This value was then compared to that of a reference SRW extract tested in the same RAST-inhibition assay. This approach assumed that the various test extracts produced RAST-inhibition curves whose slopes did not differ significantly, and this was the case in nearly each instance. We selected extract B, the candidate SRW extract, as this reference and assigned its 50% inhibition volume a value of unity. In most instances, the potency ratio for extract F, the coded duplicate of extract B, was near unity, as expected, although somewhat higher ratios were consistently obtained by laboratory no. 8 for extract F. In other laboratories, there was more variation in the potency ratio for extract F. The potency ratio for extract E should have been fivefold less than that of extract D, and this was roughly the case with most laboratories. However, laboratory no. 1 consistently identified extract E as being equally potent or more potent than extract D. Finally, for each laboratory, we calculated geometric mean potency ratios for each extract relative to extract B. These ratios are demonstrated in Table III. The mean potency ratio for extract F was approximately 1.0 for nearly all laboratories; the mean potency ratio for extract E was appreciably less than extract D in all laboratories except no. 1.

TLIEF

Eleven of the participating laboratories provided TLIEF data. Six of these laboratories use LKB Ampholine PAG plates (LKB-Produkter AB, Bromma, Sweden) (pH 3.5 to 9.5) and followed instructions supplied by LKB. The remaining five laboratories prepared their own plates in-house by use of the same pH gradient. The pH range of the TLIEF plates was measured by use of commercially prepared pI calibration kits or by eluting discs that had been punched from the gel with distilled water. A representative photograph of a Coomassie-stained gel is demonstrated in Fig. 1.

The results from the various laboratories were quite

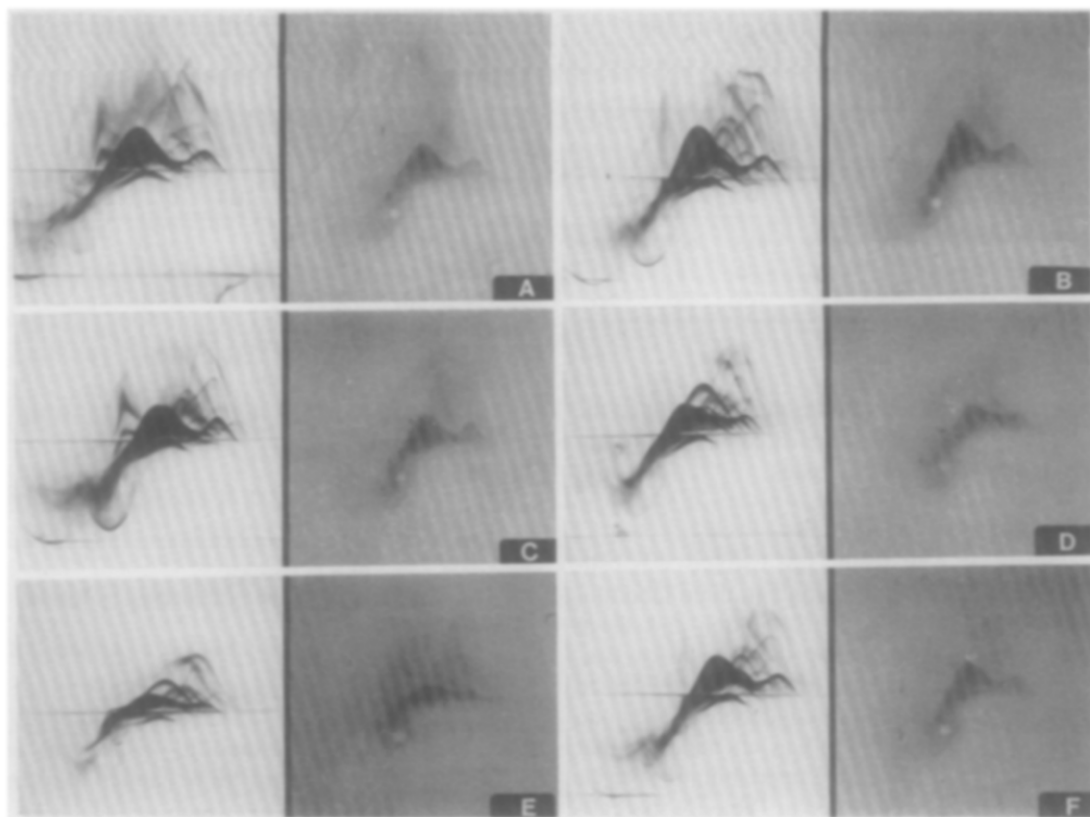


FIG. 2. CIE (*left*) and CRIE (*right*) patterns obtained for SRW extracts A through F at laboratory no. 1. Anode is to *right* in the first dimension electrophoresis and to *top* in second dimension electrophoresis. A, Extract A, 110 μ g; B, Extract B, 93 μ g; C, Extract C, 113 μ g; D, Extract D, 46 μ g; E, Extract E, 28 μ g; F, Extract F, 80 μ g. For CIE, anodal and cathodal gels contained 10 μ l of antibody per square centimeter (rabbit anti-*Ambrosia elatior* antibody from ALK). For CRIE, unstained plates were incubated in 7.5 ml serum pool from ragweed-allergic individuals diluted 1:15 for 20 hr; washed, incubated with 7.5 ml ^{125} I-labeled-anti IgE (Pharmacia) for 20 hr and autoradiographed.

similar. As expected, the banding pattern produced by the more dilute extract (E) was more faint, whereas the other test extracts produced 25 to 30 Coomassie-stained bands, mostly in the pH range of 4.0 to 6.0. Extract D contained fewer than the expected number of cathodal bands. The banding patterns produced by extracts A, B, C, and F were quite similar to one another. In laboratory no. 11 the SRW test extracts were adjusted to equal protein concentrations and retested; the banding patterns produced by the six extracts were then very similar.

CIE/CRIE

Five of the participating laboratories performed CIE, and three included CRIE assays, according to the recommended procedures provided by the International Union of Immunological Societies to the participating laboratories.⁷ All five laboratories used ALK (Allergologisk Laboratorium) rabbit antibodies to SRW for the CIE/CRIE experiments. Similar results were obtained by all five laboratories. Represen-

tative photographs from laboratory no. 1 are demonstrated in Fig. 2. CIE bands were numbered according to a previously published reference system.⁸ All six SRW extracts produced bands identified as AgE, AgK, Ag11, and Ag31 (Ra4) by visual inspection. All test extracts except D and E also produced bands identified as Ag27, Ag35 (Ra6), Ag37 (Ra5), and Ag30a. The corresponding CRIE results are also demonstrated in Fig. 2; these were run by use of the IgE-antibody pool provided. Similar patterns of radiostaining were produced by all test extracts except that the radiostaining of extracts D and E were less intense and did not contain bands corresponding to Ag27 or Ag35 (Ra6) in the case of extract E.

Laboratory no. 3 also performed CRIE experiments by use of the 10 individual IgE-containing sera comprising the IgE-antibody pool. All 10 sera contained IgE antibodies to ragweed AgE, and three of the 10 sera also contained IgE antibodies to otherwise unidentified allergens (data not shown). These data confirmed the RAST results obtained previously dur-

TABLE IV. Antigen E content (microgram per milliliter) of SRW extracts

Laboratory	SRW extract					
	A	B	C	D	E	F
6	160	108	268	125	60*	111
7	188	133	176	80	74	123
9	129	85	121	73	15	85

*Single test.

ing the preliminary investigations that showed that the IgE-antibody pool provided indeed contained IgE antibodies to relevant SRW allergens.

AgE

The content of AgE in the test extracts was estimated by radial immunodiffusion¹³ in three laboratories by use of a reference AgE solution and sheep antiserum to AgE provided by the Bureau of Biologics, FDA. One ampule of extract E provided to laboratory no. 6 could not be rehydrated so only one estimate of AgE concentration was attempted in that case. There was a considerable interlaboratory variation in test results, demonstrated in Table IV, particularly with respect to extracts C, D, and E. As expected the intralaboratory variation was not so marked, and the values obtained for the coded duplicate samples (B and F) were quite close in all laboratories.

Protein and nitrogen assays

Four laboratories provided total protein or total nitrogen assays for the six SRW extracts, and these results are demonstrated in Table V. Laboratory nos. 6 and 7 assayed total nitrogen by the Kjeldahl method, and laboratory no. 6 expressed results in terms of total protein after multiplying the total nitrogen figure by 6.25. Colorimetric assays for total protein was performed in laboratories nos. 1 (Lowry) and 11 (Bio-Rad dye binding, Bio-Rad Laboratories, Richmond, Calif.) by use of BSA as a standard. The variance in results is probably a function of normal interlaboratory variation and also differences in the analytical procedures used. Colorimetric assays have the disadvantage that certain extracts may produce colors that result in higher estimates of protein content. This may be true especially with the high values reported by laboratory no. 1 in Table V.

ELISA

Laboratory no. 3 examined the antigenic content of the SRW extracts by an ELISA inhibition assay.¹⁴ The wells of a Dynatech Microelisa plate (Synatech

TABLE V. Protein and nitrogen assays of SRW extracts

	SRW extract					
	A	B	C	D	E	F
Laboratory 1						
Protein* (mg/ml)	11.0	3.7	7.6	1.8	0.37	3.2
Laboratory 6						
Total protein†	5.75	2.0	3.75	1.19	—	2.68
Laboratory 7						
Total nitrogen‡ (mg N/ml)	1.44	1.15	0.77	0.26	0.10	0.49
Laboratory 11						
Protein§ (mg/ml)	1.40	0.74	1.31	0.60	0.10	0.77

* = Lowry assay; BSA as standard.

† = Total protein = 6.25 times total nitrogen by Kjeldahl assay.

‡ = Kjeldahl assay.

§ = Bio-Rad dye binding assay (Bio-Rad Laboratories); BSA as standard; mean of two assays.

Laboratories, Inc., Alexandria, Va.) were coated with 200 μ l of a standard SRW antigen at a concentration of 50 μ g/ml, after which residual sites were blocked with HSA and the plate was washed. To these wells were added serial threefold dilutions of each reconstituted SRW extract followed by an optimal dilution of rabbit anti-SRW antiserum (ALK). After incubation and washing, an optimal dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG was added followed by incubation, washing, and addition of enzyme substrate (p-nitrophenylphosphate). The 405 nm absorbance of each well was read. Results were expressed on a volume per volume basis by use of the recommended reconstitution volumes, and the response was converted to percentage inhibition by use of the maximum absorbance values (no inhibitor added) to represent zero inhibition; three independent assays were performed. Although relative potency of each SRW extract varied somewhat from assay to assay, as did the rank order of potencies of extracts, it was concluded that the relative potencies of extracts A, B, C, D, and F were not grossly different, whereas potency of extract E was considerably lower.

HPLC

Each of the SRW extracts was tested by size exclusion HPLC in laboratory no. 3¹⁵ by use of Toyo Soda TSK G3000 SW columns with 0.1M phosphate buffer (pH 7) and a flow rate of 0.5 ml/min. The SRW extracts were reconstituted in water, and 10 μ l samples were applied. The absorbance was recorded at 215 nm. The resulting elution profiles are demon-

strated in Fig. 3. Each extract revealed essentially the same tracing with 13 peaks at the same corresponding positions. Extract E gave a rather flattened trace, but the major peaks therein were at the same positions as for the other extracts. The HPLC assay also correctly identified extracts B and F as being duplicate samples.

Leukocyte histamine release

Laboratory no. 2 provided leukocyte histamine release data for extracts A through F by use of fresh venous blood from two ragweed-sensitive individuals. The assays were performed by use of an automatic analyzer.¹⁶ All six extracts induced greater than 50% histamine release for both individuals tested. The coded duplicates, extracts B and F, were nearly identical with respect to the dose required to induce 30% histamine release; the potency of extracts A, B, C, D, and F were not greatly different. Extract E, the diluted sample of extract D, was identified as the least potent extract in all assays. Because of the small number of individuals studied, no attempt was made to statistically analyze the data.

Intradermal skin tests

Laboratory no. 5 compared the intradermal skin test responses to the national reference extract (A) and the candidate SRW extract (B) in five SRW-allergic subjects and one nonallergic subject. Extracts A and B were reconstituted in 0.5 ml and 1.0 ml sterile distilled water, respectively, and tenfold serial dilutions were then prepared in sterile Sørensen's buffer with 0.5% HSA.¹⁰ Skin tests were placed and graded by the method of Norman¹⁷; the highest dilution demonstrating a +2 reaction was considered the end point, and the results are demonstrated in Table VI. The end points obtained in each SRW allergic subject were within a tenfold range for extracts A and B that confirmed the relative potency estimates obtained by the RAST inhibition and leukocyte histamine release assays.

STABILITY STUDIES

Sixty ampules of the lyophilized candidate SRW extract were received in December 1981, and stored at -20°C . In January 1982, a few ampules were removed from storage at -20°C and placed at constant temperature environments of $+5^{\circ}\text{C}$, $+23^{\circ}\text{C}$, $+37^{\circ}\text{C}$ and $+55^{\circ}\text{C}$. The temperature of each environment was monitored three times weekly during normal working hours. At subsequent times (2, 6, and 12 mo) a single vial from each constant temperature environment was withdrawn, visually inspected, reconstituted in 1.0 ml of 50% glycerine, diluted in serial tenfold fashion, and tested for allergenic activ-

TABLE VI. Intradermal skin test end points for the national reference extract (extract A) and the candidate SRW extract (extract B)

Subject no.	Skin test end point	
	Extract A	Extract B
SRW allergic		
1	3×10^{-10}	1×10^{-7}
2	1×10^{-7}	1×10^{-7}
3	3×10^{-9}	1×10^{-8}
4	1×10^{-7}	3×10^{-8}
5	1×10^{-8}	1×10^{-8}
Nonallergic	$>1 \times 10^{-3}$	$>1 \times 10^{-3}$

TABLE VII. Potency estimates for the candidate SRW extract after storage at various temperatures for 2, 6, and 12 mo

Storage time	Temperature				
	-20°C	$+5^{\circ}\text{C}$	$+23^{\circ}\text{C}$	$+37^{\circ}\text{C}$	$+55^{\circ}\text{C}$
2 mo	1.00	0.77	1.44	1.88	0.08
6 mo	1.00	1.47	1.22	0.94	—
12 mo	1.00	1.61	1.02	0.49	—

Potency estimates of the extracts stored at $+5^{\circ}\text{C}$, $+23^{\circ}\text{C}$, $+37^{\circ}\text{C}$, and $+55^{\circ}\text{C}$ relative to the extract stored at -20°C from individual assay.

ity (RAST inhibition), antigenic activity (CIE), and for isoelectric focusing patterns. For each assay the stabilities of extracts stored at $+5^{\circ}\text{C}$, $+23^{\circ}\text{C}$, $+37^{\circ}\text{C}$, and $+55^{\circ}\text{C}$ were compared to that of the extract stored at -20°C .

By visual inspection of the ampules, no appreciable differences were observed in the extracts stored at -20°C , $+5^{\circ}\text{C}$ and $+23^{\circ}\text{C}$ for up to 12 mo. By 6 mo the extract stored at $+55^{\circ}\text{C}$ appeared chocolate brown and baked onto the side of the glass ampule, which made reconstitution difficult. The extract stored at $+37^{\circ}\text{C}$ demonstrated similar changes by 12 mo.

The relative allergenic activity ratios are shown in Table VII. Although the potency ratios varied somewhat, demonstrating both increased and decreased activity, the ranges of values for extracts stored at $+5^{\circ}\text{C}$ and $+23^{\circ}\text{C}$ were regarded as being within the intralaboratory variance observed previously for the RAST-inhibition assay. However, a significant loss in potency of the extract stored at $+55^{\circ}\text{C}$ was observed by 2 mo, and by 6 mo the allergenic activity was virtually nonexistent. A decrease in the potency of the extract stored at $+37^{\circ}\text{C}$ was also apparent by 12 mo.

The antigenic activity after storage was tested by

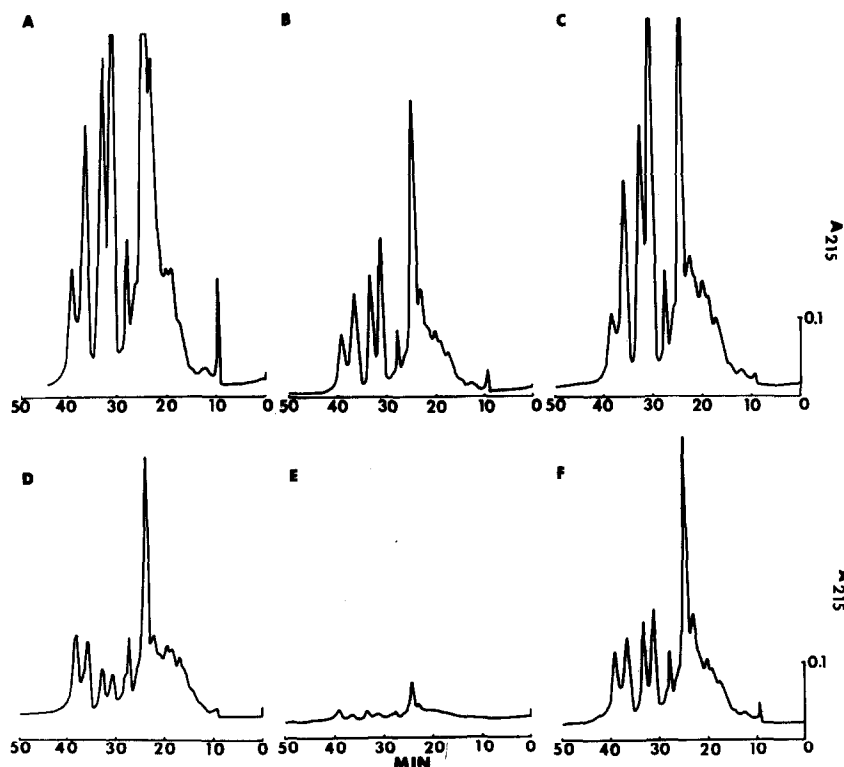


FIG. 3. Size exclusion HPLC profiles for SRW extracts, A through F at laboratory no. 3. SRW extracts were reconstituted in water and 10 microliter samples were applied. Absorbance of 215 nm is plotted on the ordinate; time is plotted on the abscissa.

CIE. The extracts stored at -20°C , $+5^{\circ}\text{C}$, and $+23^{\circ}\text{C}$ for 2 mo demonstrated minor alterations in the anodal and cathodal banding patterns, but overall the CIE patterns resembled those demonstrated in Fig. 2. After 2 mo, the extract stored at $+37^{\circ}\text{C}$ demonstrated loss of some of the cathodal bands, whereas the extract stored at $+55^{\circ}\text{C}$ demonstrated extremely diminished antigenic activity with only 1 to 2 anodal precipitin bands remaining. After 6 mo, no precipitin bands were identified in the extract stored at $+55^{\circ}\text{C}$, and the $+37^{\circ}\text{C}$ sample demonstrated no cathodal bands and fewer anodal bands. The extracts stored at -20°C , $+5^{\circ}\text{C}$, and $+23^{\circ}\text{C}$ were stable and demonstrated patterns similar to those obtained after 2 mo in storage. After 12 mo, only the extracts stored at -20°C , $+5^{\circ}\text{C}$, and $+23^{\circ}\text{C}$ demonstrated stable CIE patterns; the extract stored at $+37^{\circ}\text{C}$ demonstrated only 4 to 5 distinguishable bands, whereas the extract stored at $+55^{\circ}\text{C}$ demonstrated no antigenic activity.

The TLIEF patterns correlated well with the antigenic banding patterns observed in the CIE study. The extracts stored at -20°C , $+5^{\circ}\text{C}$, and $+23^{\circ}\text{C}$ continued to demonstrate sharp banding patterns in the pH 4 to 6 range for the entire 12-month study interval. The extract stored at $+55^{\circ}\text{C}$ had lost all but

a single identifiable band by 12 mo, whereas the extract stored at $+37^{\circ}\text{C}$ demonstrated considerable loss of bands and smearing of the bands by 12 mos.

DISCUSSION

The data from the preliminary investigations indicated that the candidate SRW extract compared favorably to existing national and local reference SRW extracts in terms of allergenic, antigenic, and protein content. These findings were confirmed and extended in the subsequent international collaborative study involving several additional laboratories and several companion SRW test extracts. By use of a variety of physicochemical and immunochemical procedures, it was demonstrated that the candidate SRW extract could be used to assign a relative potency to other SRW extracts. For the RAST-inhibition assay, data have been obtained to estimate the intralaboratory and interlaboratory variance in such assigned potency estimates. By use of this technique, participating laboratories were able, for the most part, to identify a companion test extract where the allergenic potency was fivefold lower than the other extracts. By performing multiple RAST-inhibition assays, the participating laboratories were usually able to identify the coded

duplicate samples (extract B and F) as being of equal allergenic potency. Finally, the stability studies performed indicated that the candidate SRW extract maintained allergenic activity for at least 12 mo when it was stored at either -20°C or $+5^{\circ}\text{C}$.

After completion of the multinational collaborative study, the data were submitted to the WHO, who subsequently accepted the candidate SRW extract as an International Reference Standard with an arbitrary international unitage of 100,000 per ampule. This number is chosen so that all assays with dilutions up to 10^{-5} , such as RAST inhibition, will still reveal simple whole numbers of international units.

Note added in proof. The stability of the lyophilized reference standard stored at -20°C or $+5^{\circ}\text{C}$ for 24 mo has recently been documented.

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APPENDIX

Laboratories participating in the collaborative study

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