

Original articles

Production of a proposed International Reference Standard *Alternaria* extract

II. Results of a collaborative trial

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A lyophilized Alternaria extract prepared from a defined eight-strain source material was compared with three other Alternaria extracts to assess its potential suitability as an international standard (IS). Twenty-two laboratories in 12 different countries collaborated. Assay methods included RAST inhibition, quantitative immunoelectrophoresis, thin-layer isoelectric focusing, skin testing, leukocyte histamine release, and various other methods for thorough characterization of the extracts. In addition, three laboratories quantitated specific allergen in each extract. The proposed IS extract could be used to assign a relative potency to other test extracts. In separate studies, the proposed IS extract was demonstrated to be stable for at least 21 months when it is stored at -70°C , -20°C , and 4°C . (J ALLERGY CLIN IMMUNOL 1988;81:651-63.)

In 1981 the Allergen Standardization Subcommittee of the IUIS initiated a program of allergen standardization to establish several allergen extracts that would meet WHO specifications as IS. After the successful production, testing, and acceptance of *Ambrosia elatior*,¹ *Phleum pratense*,² and *Dermatophagoides pteronyssinus*³ extracts as IS, a second group of allergens, including *Alternaria*, birch pollen, Bermuda-grass pollen, cat, and dog were selected for production and study.

An *Alternaria* Working Group (Appendix I) prepared specifications for candidate *Alternaria* IS preparations

that were circulated to manufacturers interested in participating. The ARRL at Mayo Clinic, Rochester, Minn., acted as the coordinating center. Six *Alternaria* extracts were submitted, accompanied by samples of source materials used to prepare the extract. Each extract was coded and distributed to laboratories of the *Alternaria* Working Group for preliminary study; source strain materials were also coded and shipped to an expert *Alternaria* taxonomist for independent identification. Studies conducted by the *Alternaria* Working Group and the results of the source material identifications were shared among the group

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Abbreviations used

IS:	International standard(s)
IUIS:	International Union of Immunological Societies
ARRL:	Allergen Resource Reference Laboratory
WHO:	World Health Organization
ATCC:	American-Type Culture Collection
CIE:	Crossed immunoelectrophoresis
CRIE:	Crossed radioimmunoelectrophoresis
TLIEF:	Thin-layer isoelectric focusing
GM:	Geometric mean
GVC:	Geometric coefficient of variation
QIE:	Quantitative immunoelectrophoresis
RIE:	Rocket immunoelectrophoresis
SRID:	Single radial immunodiffusion
BSA:	Bovine serum albumin
PBS:	Phosphate-buffered saline
HPLC:	High-performance liquid chromatography
SDS-PAGE:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
HSA:	Human serum albumin
LHR:	Leukocyte histamine release

to select a suitable extract to be recommended as the proposed IS. This preliminary work was reported to the Steering Committee of the Allergen Standardization Subcommittee of the IUIS and is published elsewhere.⁴ An eight-strain extract was chosen as the proposed IS based on (1) adherence to written specifications, (2) independent verification of source material identity, and (3) documented content of *Alternaria* allergenic, antigenic, and specific allergen activity. Subsequently, the proposed *Alternaria* IS was tested according to WHO guidelines⁵ in an international collaborative study in parallel with three other coded *Alternaria* extracts. This article describes the results obtained in this collaborative study; we demonstrate that the proposed *Alternaria* IS can be used to assign an arbitrary unitage of potency to other *Alternaria* extracts.

MATERIAL AND METHODS

The lyophilized *Alternaria* extracts used for the collaborative study are listed in Table I. These included coded duplicates of the proposed *Alternaria* IS as well as *Alternaria* extracts previously supplied by commercial manufacturers as candidate extracts.

Extracts

The various extracts were previously described in detail.⁴ Briefly, the collaborative study extracts were as follows.

TABLE I. *Alternaria* extracts used in the collaborative study

Collaborative study code letter	Identification	Preliminary study ^a code No.
A	Candidate <i>Alternaria</i> extract	6
D	Commercial <i>Alternaria</i> extract*	3
L	Commercial <i>Alternaria</i> extract*	5
M	Coded duplicate of extract A	6
S	Commercial <i>Alternaria</i> extract†	1

*Prepared previously as candidate *Alternaria* extracts.

The candidate IS. This extract was coded A and M and corresponded to extract No. 6.⁴ The extract was prepared from eight *Alternaria* strains grown by the manufacturer on synthetic medium. All strains were demonstrated to sporulate, and a mixture of mycelia and spores was extracted, purified by ultrafiltration, and lyophilized. Metabolic antigens were assumed to be removed by ultrafiltration.

Extract D. This extract was prepared from four different source strains received by the manufacturer from various commercial sources and corresponded to extract No. 3.⁴ Active sporulating cultures were killed, and the mycelial mat with spores were extracted, clarified by centrifugation, and dialyzed. After removal of mycelium and spores, the spent culture medium was also clarified and dialyzed. The mycelial/spore extract and spent culture media were then mixed and lyophilized.

Extract L. This extract was prepared from a single *Alternaria* strain grown on synthetic medium by an independent supplier and corresponded to extract No. 5.⁴ A mixture of mycelia and spores was extracted, filter sterilized, and lyophilized.

Extract S. This extract was prepared from a single strain of *Alternaria* grown and extracted by the manufacturer and corresponded to extract No. 1.⁴ Raw material, consisting of a mycelial mat and spores, was harvested, washed with acetone, crumbled, dried, ball-milled, and tested for viability before extraction. The extract was then sterile filtered and vialled.

All extracts (500 ml or equivalent) were shipped directly to ATCC, Rockville, Md., where they were dispensed into glass ampules, lyophilized, and sealed. The lyophilized extracts met U.S. federal specifications and regulations for general sterility tests as outlined in section 610.12 of Title 21 of the Code of Federal Regulations.

***Alternaria* RAST reagents**

Solid-phase reagent. For purposes of the collaborative study, participants were supplied with *Alternaria* paper disks (batch No. 9469) kindly donated by Pharmacia AB, Uppsala, Sweden.

IgE antibody pool. The coordinating center collected an IgE antibody pool containing equal parts of defibrinated delipidated plasma from eight subjects with typical clinical histories of *Alternaria* sensitivity who had not previously received *Alternaria* immunotherapy. The IgE antibody levels of serum specimens from each donor were measured by RAST with *Alternaria*-coupled microcrystalline cellulose prepared in the ARRL. Plasma donors were in good health, and each plasma was free of antibody to human immunodeficiency virus and of hepatitis B surface antigen. After defibrination by dialysis against Ca^{++} -containing buffers, excess Ca^{++} was removed by dialysis against 0.15 mol/L of NaCl; the plasmas were then delipidated by Freon-113 treatment with a ratio of two parts plasma to one part Freon.⁶ The pooled sera were shipped frozen to ATCC where they were sterile filtered into 1.0-ml aliquots, lyophilized, and sealed in glass ampules. Samples of serum specimens from each individual used in the antibody pool were retained at the coordinating center.

Participants

An international collaborative study involving the proposed *Alternaria* IS was organized in accordance with published guidelines.⁵ Invitations were sent to approximately 30 manufacturers, regulatory agencies, and research investigators. Because of limited supplies of some reagents, we limited the number of participants to 22 (Appendix II). All laboratories were assigned a code number; the order in which they are listed in the appendix does not correspond to the code number.

Conduct of the study

Participants were urged to evaluate the *Alternaria* extracts by use of their laboratory assay methods, which included "standard" procedures agreed to previously by Allergen Standardization Subcommittee members. Ampules of *Alternaria* IgE antibody pool, individual IgE-containing sera, and *Alternaria* paper disks were provided on request. Data sheets for RAST inhibition, CIE/CRIE, and TLIEF were provided. Participants were requested to return their raw data together with their interpretation of the results.

Statistical treatment

The RAST-inhibition data provided by participating laboratories were analyzed by a computerized version of a parallel-line bioassay method.⁷ The inhibition curve of each individual *Alternaria* extract was analyzed by polynomial regression analysis that included at least four data points in the range of 10% to 90% inhibition, which bracketed 50%, to elicit a correlation coefficient of >0.90 . Inhibition curves of extracts failing to meet the above criteria were considered invalid. Parallelism between inhibition curves was tested by Student's *t* test by use of the proposed IS (extract A) as the reference standard; probability values of <0.01 were

TABLE II. Summary of methods used in collaborative study

Method	No. Laboratory	No. Assay
Physicochemical		
Dry weight	3	10
Residual moisture	2	2
Protein	9	10
TLIEF	15	24
HPLC	5	9
SDS-PAGE/ Western blot	3	5
Immunochemical	16	50
RAST inhibition		
CIE/CRIE	13	25
Biologic		
Quantitative skin test	2	11
LHR	2	6
Others*	6	16

*Direct RAST, RIE, HPLC/RAST, SRID, TLIEF/immunoblot, TLIEF/IE, TLIEF/RIE, and TLIEF/RAST.

considered to be statistically significant. When inhibition curves of extract A failed to meet the validity criteria listed, the entire assay was considered invalid. The GM and GCV⁸ for each extract were calculated for each laboratory from the individual assays provided. A potency ratio was then determined with extract A as the reference standard. Finally, an overall GM was calculated from the potency ratios from each individual laboratory to assign a relative potency value to each extract.

Assay methods

The methods used in the study are summarized in Table II. The coordinating center recommended reconstitution volumes and dilutions for some of the assays; however, the participants were free to vary these recommendations according to their needs.

RESULTS

RAST inhibition/direct RAST

The techniques used for RAST inhibition were primarily versions of published methods.⁹ Sixteen laboratories provided RAST-inhibition data. Seven laboratories used both the Pharmacia paper disks and IgE antibody pool provided by the coordinating center. Five laboratories used *Alternaria* extracts coupled to solid-phase supports and an antibody pool containing IgE prepared in their respective laboratories. Three laboratories used an *Alternaria* extract coupled to solid-phase supports prepared in their laboratory and the IgE-containing antibody pool provided by the coordinating center. Another laboratory used an *Alternaria* extract coupled to paper disks prepared in their

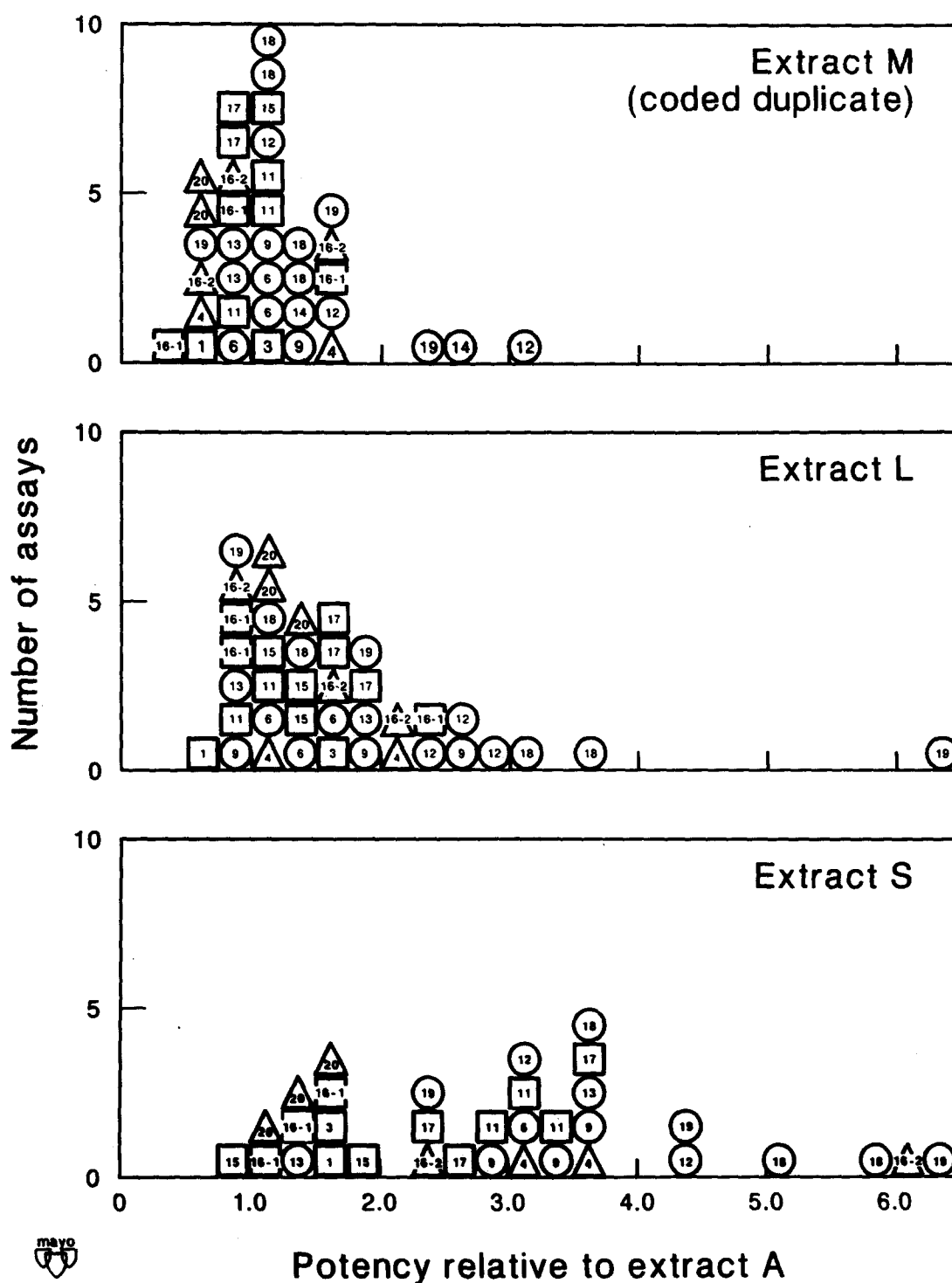


FIG. 1. Distribution of potency estimates relative to extract A for individual RAST-inhibition assays. Each symbol represents an assay result, and the numbers refer to the coded laboratory. The symbols designate the origin of the RAST reagents; in-house reagents ($\square$); coordinating center reagents ($\circ$); in-house solid-phase and coordinating center IgE antibody pool reagents (Δ).

laboratory and compared an IgE antibody pool from their laboratory to that of the IgE antibody pool supplied by the coordinating center. Thirteen of the laboratories used commercial radiolabeled anti-IgE, and

three laboratories used radiolabeled anti-IgE prepared in their respective laboratories. Each laboratory that used reagents prepared in their respective laboratory provided information regarding the preparation of

these reagents. Thirteen of 16 laboratories reconstituted the lyophilized *Alternaria* extracts in the recommended volume (500 μ l per ampule). Of the 13 laboratories, eight prepared serial threefold dilutions and tested aliquots in their RAST-inhibition assays, as suggested by the coordinating center; the remaining laboratories used their own reconstituted volumes and dilutions. In general, the slopes of the inhibition curves of extracts A, M, L, and S were similar. Extract D failed to produce >30% inhibition in most assays. The results from valid assays from individual laboratories for extracts M, L, and S are illustrated in Fig. 1.

The relative potency values of valid assays for extracts M, L, and S calculated from each laboratory are presented in Table III. No valid assays were obtained for extract D. Extract A was used as the reference standard and assigned a potency value of unity. In most cases, the potency ratio of extract M, the coded duplicate of extract A, was near unity. For the most part, the extracts were ranked $S > L > M = A$. Laboratory No. 15 found a different rank order ($L > S > M = A$). Laboratory No. 14 failed to obtain valid assays for extracts L and S; therefore, no rank order could be assigned.

Laboratory No. 3 performed direct RAST analysis of the five *Alternaria* extracts. Three ampules of each extract were reconstituted in a total of 1.0 ml of RAST diluent. CMBR-activated paper disks were reacted with 50 μ l of each coded extract, and the percentage binding by an antibody pool containing IgE antibody to *Alternaria* prepared in their laboratory was determined. The binding percentages were 19.6%, 19.1%, 13.2%, 10.3%, and 1.6% for extracts M, A, L, S, and D, respectively.

QIE

The methods used for CIE/CRIE have been described previously.¹⁰ Eight laboratories used commercial rabbit anti-*Alternaria* (ALK, Copenhagen, Denmark), whereas the remaining laboratories used rabbit anti-*Alternaria* prepared in their respective laboratory as the CIE developing antibody. One laboratory compared two different rabbit anti-*Alternaria* antibody preparations raised from two different *A. tenuis* antigen preparations for CIE analysis. Five laboratories used a human allergic serum pool prepared in their respective laboratories, five used only the IgE antibody pool supplied by the coordinating center, and two laboratories used both the pooled and individual sera supplied by the coordinating center for CRIE analysis. One laboratory provided only CIE data. Similar results were obtained by all laboratories, and representative photographs from laboratory No. 17 are illustrated in Fig. 2. For the proposed IS (extracts A

TABLE III. RAST-inhibition potency ratios of unknown extracts relative to extract A

Lab	Relative potency		
	M = A	L	S
1	0.55*	0.71	1.56
3	1.06	1.69	1.70
4	1.08 (77)†	1.54 (47)	3.39 (9)
5	—	—	—
6	0.95 (9)	1.31 (19)	3.20
9	1.20 (9)	1.53 (85)	3.21 (15)
11	0.94 (17)	0.95 (15)	3.13 (5)
12	1.74 (72)	2.52 (10)	3.70 (27)
13	0.84 (6)	1.21 (68)	2.10 (107)
14	1.89 (57)	—	—
15	1.08	1.42 (6)	1.34
16-1	0.86 (79)	1.24 (74)	1.31 (25)
16-2	0.93 (63)	1.43 (59)	3.92 (90)
17	0.91 (9)	1.67 (4)	2.81 (23)
18	1.23 (16)	2.06 (71)	4.70 (28)
19	1.31 (97)	2.23 (65)	4.04 (62)
20	0.59 (26)	1.15 (15)	1.31 (27)
Overall GM	1.02	1.44	2.52
Overall GCV (percent)	38	38	57
No. laboratories	16	15	15
No. determination	37	39	34

Lab = laboratory.

*GM.

†GCV (percent).

and M) 10 to 20 precipitin bands were observed. Depending on the length of exposure and the serum used for autoradiography, from two to approximately 10 of these bands could be demonstrated to bind IgE by use of CRIE. The most intensely radiostaining peak, which produced a diffuse precipitate throughout the anodal gel, masked smaller allergen bands in many instances. In most assays performed, extract D failed to demonstrate either precipitating (antigens) or IgE-binding (allergen) bands. The largest number of bands was detected in the extract with the highest protein concentration, extract S.

Laboratory No. 15 quantitated the major allergenic content of the extracts with radiostained RIE.¹¹ Each extract was reconstituted with 250 μ l of buffer, and 5- μ l samples of *Alternaria* extracts were applied in duplicate to 1.0-mm thick antibody-containing gels (150 to 600 μ l of ALK antibody) and electrophoresed for 18 hours at 2 V/cm at 18°C. Unstained RIE plates were incubated overnight with an antibody pool containing IgE to *Alternaria* prepared in their laboratory, followed by incubation with radiolabeled anti-IgE

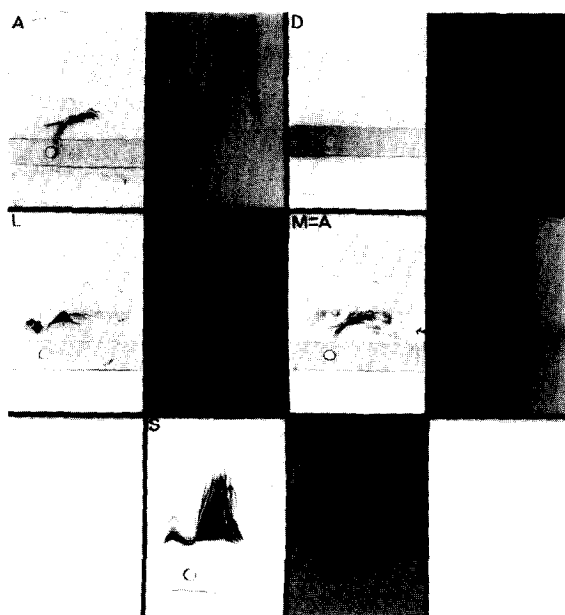


FIG. 2. CIE (left) and CRIE (right) patterns obtained for *Alternaria* extracts A, D, L, M, and S at laboratory No. 17. Anode is to the right in the first dimension electrophoresis and top in the second dimension electrophoresis. Extracts were reconstituted in 250 μ l of distilled H₂O, and 14 μ l each was applied. For CIE, gels contained 50 μ l/ml of agarose rabbit anti-*A. iridis* (ALK, Copenhagen). For CRIE, unstained CIE plates were incubated with in-house *Alternaria* IgE antibody pool (15 patients) diluted 1:24 for 18 hours, washed, incubated with ¹²⁵I-labeled anti-IgE (Pharmacia) for 18 hours, and autoradiographed for 4 weeks at -20° C.

(Pharmacia). The relative contents of the dominating allergen, based on degree of radiostaining, were calculated on the basis of mean heights of duplicate rockers with extract A as the reference. No allergen was detected in extract D, and extracts L, M, and S had mean values of 1.12, 0.82, and 1.33, respectively.

Specific allergens

Three laboratories (Nos. 4, 5, and 6) determined the concentrations of specific allergens in all five *Alternaria* extracts. Laboratory No. 4 identified the content of Alt-I¹² on CRIE films in which an intermediate gel containing 1 μ l/cm² of monospecific rabbit anti-*Alternaria* Ag 1 antibody was inserted in the anodal portion of the gel. All extracts except extract D contained Alt-I. Laboratory No. 4 used monospecific rabbit antibodies for Alt-I,¹² and Ag B* in RIE and SRID quantification analyses. Assays for those specific allergens demonstrated the presence of Alt-I and Ag B

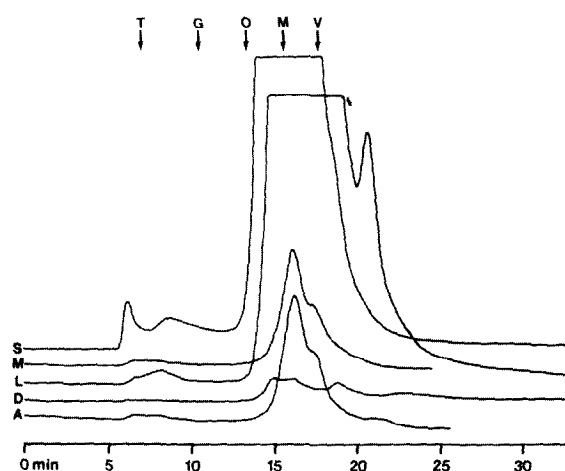


FIG. 3. Size exclusion HPLC profiles for *Alternaria* extracts A, D, L, M, and S at laboratory No. 17. The extracts were dissolved in 500 μ l of distilled water, and 20- μ l samples were applied. Absorbance was measured at 254 nm at a flow rate of 0.9 ml/min in a Bio Sil TSK 250 column connected with a guard column (Bio Rad Laboratories) equilibrated with 0.5 mol/L of ammonium acetate, pH 6.5. Absorbance is plotted on the ordinate, and time is plotted on the abscissa. The arrows indicate retention times of calibration materials: thyroglobulin (T, 670,000 daltons), gamma globulin (G, 158,000 daltons), ovalbumin (O, 44,000 daltons), myoglobin (M, 17,000 daltons), and vitamin B₁₂ (V, 1350 daltons).

(Table IV); neither of these specific allergens was detected in extracts D, and Ag B was not present in extract L.

Laboratory No. 5 determined the concentration of specific allergen in the five *Alternaria* extracts by RIE by use of a monospecific antiserum to allergen Ag 8.¹³ All ampules were reconstituted in 500 μ l of distilled H₂O. Extract A (3, 5, and 10 μ l) was used as the reference standard, and 5 μ l of the other extracts was applied to the gel containing 500 μ l of the monospecific antiserum. Relative potency determinations of Ag 8 content, with extract A as the reference, were L, 170%; M, 100%; and S, 200%. The Ag 8 content of extract D was not determined because of a non-specific precipitation.

Laboratory No. 6 quantitated the relative content of Alt-I with Alt-I RAST-inhibition assays.¹⁴ Relative potency determinations of Alt-I content with A as the reference were L, 149%; M, 82%; and S, 184%. No Alt-I could be detected in extract D. A summary of the QIE data provided by 13 participating laboratories is presented in Table IV.

TLIEF

Twelve laboratories used LKB Ampholine PAGE-plates (pH 3.5 to 9.5) (LKB-Produkter AB, Bromma,

*Nyholm L, Løwenstein H. Unpublished results.

TABLE IV. Summary of QIE results obtained in participating laboratories

Sample	No. of Laboratories								
	13	12	1	1	1	1	1	1	1
	CIE (No. bands)	CRIE	Alt-I (CRIE %)	Alt-I (RIE %)	Alt-I (SRID %)	Ag B (RIE %)	Ag B (SRID %)	Ag 8 (RIE %)	Alt-I (ALT-I RAST-I %)
A, reference	9-20	1-10	100	100	100	100	100	100	100
D	0-5	0	0	0	0	0	0	NP	0
L	5-12	1-4	NM	143	130	0	0	170	149
M = A	9-20	1-10	89	110	124	87	89	100	82
S	9-27	1-6	NM	167	130	1654	1584	200	184

NP = nonspecific precipitation; NM = not measurable, precipitation arc ran out of gel.

Sweden) following recommended instructions, and three laboratories prepared their own isoelectric focusing gels. Focusing patterns were very similar; however, laboratories that used silver-staining methods consistently demonstrated more protein bands than those observed with Coomassie blue-staining methods. The results obtained by participants in the collaborative study were identical to results illustrated in the preliminary study.⁴ Most bands were located in the pH 4.0 to 6.0 range, with fewer bands appearing in the cathodal region. When ampules were reconstituted and tested with equal volumes of samples, extracts S and L demonstrated the largest number of components because of their higher protein concentrations. Extracts A and M (coded duplicate proposed IS) demonstrated fewer bands, but all participants regarded A and M as similar, if they were not identical. Extract D did not demonstrate any Coomassie blue-stained bands and demonstrated bands in the silver-stain procedures only when higher concentrations were tested.

Laboratory No. 19 determined IgE binding proteins by an immunoblotting procedure that involved transfer of the focused bands from the gel to a nitrocellulose membrane. The membrane was then incubated with 2% BSA, 2% horse serum in PBS, followed by incubation with an *Alternaria*-specific IgE serum pool prepared in their laboratory diluted 1:5 in the above buffer. The membrane was washed, incubated with radiolabeled anti-IgE (Pharmacia), washed again, and autoradiographed for 4 days. All the extracts, except extract D, demonstrated IgE binding in the pH 4.0 to 4.5 region; however, extract L also demonstrated an IgE-binding band in the basic region of the gel (results not presented). Extracts S, M, and A were demonstrated to have this cathodal component but in a much reduced concentration. Laboratory No. 13 prepared 7% polyacrylamide gels (pH 3 to 10), applying the

samples in duplicate. One set was stained, whereas the other was cut into 1 by 0.5-cm strips and eluted with PBS. The eluted fractions were then coupled to CNBr-activated paper disks for determining allergenic activity by RAST. All extracts, except extract D, demonstrated increased RAST activity in the pH 3.5 to 5.0 region of the gel, as compared to activity observed in other regions of the gel, (results not presented). Extract L did demonstrate some activity in the pH 8.5 to 10 region of the gel.

With a combination of isoelectric focusing and immunoelectrophoresis, laboratory No. 15 was able to demonstrate the pH distribution of *Alternaria* antigens in the five coded extracts. Coomassie blue-stained precipitates were detected in the pH intervals 3.5 to 5.2, 5.2 to 7.0, and 8.0 to 8.2. Corresponding autoradiographic analysis with an antibody pool containing IgE and radiolabeled anti-IgE prepared in their laboratory demonstrated heavy radiostaining for extracts A, L, M, and S in the pH interval 3.5 to 5.2; some radiostaining was also noted for extract D. In all cases, at least three allergens could be detected (pH 3.5 to 5.2) with additional radiostaining in the pH interval 8.0 to 8.2 for extract S (results not presented).

HPLC

The *Alternaria* extracts were analyzed by size exclusion HPLC in five laboratories. A representative composite tracing is illustrated in Fig. 3. Three laboratories used size-exclusion columns (TSK G 2000 SW, Bio-Rad Laboratories, Inc., Richmond, Calif.) with phosphate buffers (0.1-0.2 mol/L, pH 7.0). The flow rate varied from 0.5 ml/min to 1.0 ml/min. Absorbance was recorded at 280 nm. The extracts were reconstituted in distilled water (100 to 250 μ l per ampule) and either prefiltered through a 0.45- μ m filter or passed through a precolumn (TSK SW pre-

TABLE VA. Physicochemical properties of *Alternaria* extracts

Extract	Protein content (mg/ampule)									
	Laboratory No.									
	1*	2†	4‡	6§	11*	13‡	13*	14	15¶	16§
A	0.20	0.31	0.35	0.11	0.09	0.29	0.10	0.04	0.10	0.25
D	<0.05	0.30	0.54	0.10	<0.01	0.51	<0.01	<0.01	0.04	0.23
L	0.45	3.65	3.60	0.76	0.17	3.70	0.22	0.08	0.14	1.68
M	0.25	0.32	0.32	0.11	0.09	0.26	0.10	0.05	0.08	0.30
S	2.53	12.70	7.10	3.66	0.60	6.06	0.75	0.26	0.36	1.09

*BioRad Coomassie blue G assay.

†Ninhydrin assay.

‡Lowry assay.

§Amino acid assay.

||Bradford assay.

¶Modified Lowry assay.¹⁶**TABLE VB.** Physicochemical properties of *Alternaria* extracts

Extract	Laboratory No.				
	Dry weight (mg/ampule)			Residual moisture (%)	
	2	3	11	2	3
A	2.0 ± 0.20 (n = 5)	2.8	2.80 (16%)*	4.1 ± 1.0 (n = 3)	QNS
D	0.6 ± 0.03 (n = 3)	0.5	1.37 (32%)	3.9 ± 1.0 (n = 2)	QNS
L	19.8 ± 1.10 (n = 3)	16.8	20.96 (14%)	1.5 ± 0.4 (n = 3)	QNS
M	1.9 ± 0.30 (n = 3)	1.1	2.91 (15%)	2.5 ± 1.2 (n = 3)	QNS
S	29.5 ± 1.90 (n = 3)	27.2	29.58 (2%)	0.5 ± 0.1 (n = 3)	2.68

QNS = quantities supplied were not sufficient for testing.

*Coefficient of variation for six determinations.

column). Injection volumes varied from 90 to 100 μ l per extract. Laboratory No. 13 made independent runs of the extracts in sizing columns TSK 3000 SW and TSK 2000 SW with phosphate buffer, pH 6.8; injection samples of 10 μ l were used. TSK 3000 SW column fractions were added to CNBr-activated paper disks (75 μ l per disk), and RAST activity was determined with the IgE-containing antibody pool from the coordinating center and a radiolabeled goat antihuman IgE prepared in their laboratory. The RAST activity, except for extract D, was found in the early fractions, corresponding to high-molecular-weight components (data not presented).

Physicochemical determinations

Seven laboratories provided total protein assays based on colorimetric analyses (Table VA). Two laboratories used a Bio-Rad dye binding assay (Bio-Rad Laboratories, Richmond, Calif.), one used Lowry protein assay,¹⁵ one used a modified Lowry protein assay,¹⁶ one used both the Bio-Rad dye binding assay

and the Lowry protein assay, one used the Bradford assay,¹⁷ and one used the ninhydrin method. Two laboratories determined protein weight by amino acid analysis. In laboratory No. 6, the samples were hydrolyzed in 6 N of HCl at 110° C for 24 hours; amino acids were then derivatized to their phenylthiocarbonyl derivatives and separated on a Beckman (Beckman Instruments, Inc., Irvine, Calif.) C₁₈ column (4.6 mm by 150 mm) heated at 30° C.¹⁸ The figures in Table VA represent average total protein weight calculated with peak area and peak height based on standards. In laboratory No. 16, the total protein content in the *Alternaria* preparations was determined by an automatic recording amino acid analyzer.¹⁹ The samples were hydrolyzed in 6 mol/L of HCl at 110° C for 24 hours. The amino acids were then separated by liquid chromatography on a sulfonated polystyrene cation exchange resin and subsequently determined continuously and quantitatively by colorimetry based on the Ninhydrin reaction.

Each laboratory expressed data in milligrams per

ampule relative to the volume of reconstitution, dilution, and their protein standard (BSA and bovine gamma globulin). The rank order was $S > L > M = A > D$ in seven assays, $S > L > D > M = A$ in two, and $L > S > M = A > D$ in one assay.

Three laboratories provided dry weight per ampule determinations that were in good agreement (Table VB). Laboratories Nos. 2 and 3 provided residual moisture measurements.

SDS-PAGE/Western blotting

Three laboratories analyzed the five *Alternaria* extracts by SDS-PAGE and blotting onto nitrocellulose membranes with Western blotting methods. Laboratory No. 1 used SDS-PAGE²⁰ as their resolving gel. Each extract was reconstituted in 250 μ l 0.9% NaCl; aliquots of each sample were diluted 1:2 with two times treatment buffer (0.125 mol/L Tris HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 2 mg/ml of bromophenol blue), and approximately 35 to 45 μ l of each sample was electrophoresed in duplicate at 30 mA per gel until bromophenol blue was approximately 1 cm from the bottom of the gel. One gel was then stained for protein with Coomassie blue. The number of stained bands ranged from 0 (extract D) to 30 (extract S). The other gel was transferred electrophoretically to a nitrocellulose membrane for Western blotting analysis with an in-house IgE antibody-containing serum pool.²¹ All samples, with the exception of extract D, demonstrated IgE binding to high-molecular-weight components migrating approximately 0.5 to 0.6 cm from the origin (results not presented). Extracts S and L demonstrated considerable differences in autoradiographic patterns compared to extracts M and A, probably because of the higher protein contents in the former samples. At apparent molecular weights of 35,000 or less (approximately 4.4 cm from the origin), the IgE binding appeared unique for each extract.

Laboratory No. 11 analyzed the extracts by electrophoresis under nonreducing conditions in SDS-PAGE (12.5%).²⁰ Each extract was dissolved in 100 μ l of distilled H₂O, further diluted 1:2 with sample application buffer, and 50 μ l of each extract was tested in duplicate. One gel was stained with Coomassie blue R250 (Fig. 4, A), and the other gel was electrophoretically blotted.²² The proteins transferred onto nitrocellulose membrane were blocked with BSA, and the sheet was then incubated with serum from an individual known to be *Alternaria* sensitive. The sheet was washed and incubated with radioiodinated anti-IgE (Pharmacia), washed again, and autoradiographed (Fig. 4, B). The protein-stained gel demonstrated faint staining. There was marked similarity between extracts A and M, but marked differences among the

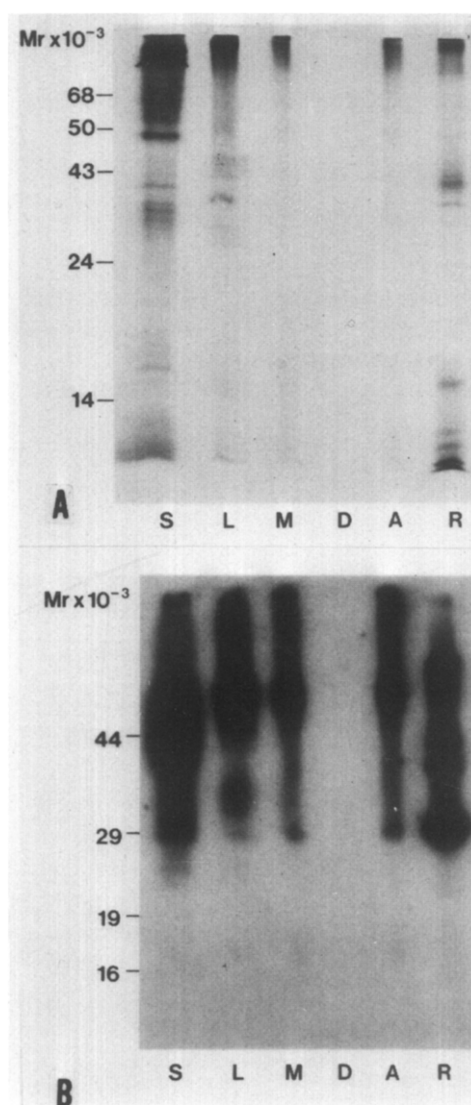


FIG. 4. SDS-PAGE/Western blotting for *Alternaria* extracts A, D, L, M, and S at laboratory No. 11. Extracts were dissolved in 100 μ l of distilled H₂O and diluted 1:2 with sample application buffer (nonreducing), and 50 μ l was loaded per lane. **A**, One gel was stained with Coomassie blue. **B**, The other gel was electrophoretically blotted onto nitrocellulose, blocked with BSA, incubated with in-house IgE antibody to *A. alternata*, washed, incubated with ¹²⁵I-anti-IgE (Pharmacia), and autoradiographed. Molecular weight markers are designated to the left and the lanes with corresponding extracts at the bottom of each photograph. R, laboratory No. 11 reference preparation.

others. No bands were evident in extract D. It was noted that proteins with apparent molecular weight of >100,000 would not run into the gel. The allergen profile technique demonstrated both quantitative and qualitative differences for extracts L and S; however, extracts A and M were identical. Again extract D demonstrated no detectable bands.

Biologic activity

Two laboratories measured the biologic activity of the *Alternaria* extracts by quantitative skin test.²³ For end point puncture skin tests, six *Alternaria*-sensitive individuals and one non-*Alternaria*-sensitive atopic individual were tested at laboratory No. 1. Ampules of the candidate extract were reconstituted with 500 μ l of NaCl-HSA diluent, and serial tenfold dilutions were prepared. The rank order of potency, $S > L > A > M > D$, was determined by measuring the area (square millimeters) of the wheal and flare as well as the diameters of each. Three *Alternaria*-sensitive individuals and one nonatopic individual were tested at laboratory No. 6. Ampules of the extracts were reconstituted in 500 μ l of Sorensen buffer with 0.5% HSA, and serial tenfold dilutions were prepared. Skin tests were placed and graded according to the method of Norman²⁴; the highest dilution eliciting a 2+ reaction was considered the end point. The rank order of potency was the same as obtained in laboratory No. 1.

Two laboratories measured the biologic activity of the five *Alternaria* extracts by LHR with fresh venous blood from three *Alternaria*-sensitive patients. In each center, the extracts were reconstituted in 500 μ l of diluent (0.85% NaCl or Sorensen's buffer with 0.5% HSA), and serial tenfold dilutions were prepared. Dilutions of anti-IgE served as positive controls. Extracts A, L, M, and S produced significant histamine release (>20%) from the cells of all *Alternaria*-sensitive patients tested. No histamine release was produced with extract D.

Stability studies

Accelerated stability studies of the proposed IS were initiated by incubating ampules at several ambient temperatures (-70° , -20° , 4° , 25° , 37° , and 56° C). After 21 months, ampules stored at 37° and 56° C lost 90% of their allergenic activity, as measured by RAST inhibition, lost nearly half of the recognizable Coomassie blue-stained bands by CIE, and demonstrated marked smearing and loss of band identity by TLIEF. Less marked changes were noted for extracts stored at 25° C. Three- and 4-year repeat stability studies are planned.

Approximately 5% of the ampules of the proposed IS stored at ATCC exhibited appreciable shrinkage in contents by visual inspection 1 year after production. The dry weights of these samples did not differ. No significant differences in allergenic activity (RAST inhibition) could be demonstrated between "shrunken" and normal-appearing extract contents in the ampules. Ampules that appeared to have visually less material were set aside and not used in the collaborative study and will not be distributed in the future. The entire

inventory was reinspected in September 1986; no additional changes in appearance were noted.

DISCUSSION

The preliminary data from members of the *Alternaria* Working Group indicated that a proposed IS that met the specifications outlined could be selected from the candidate extracts submitted.⁴ The proposed IS (extract M = A) was further tested by subsequent international collaborative study and found to be representative of most companion test extracts. The most widely used assay methods, RAST inhibition, TLIEF, and CIE/CRIE, clearly demonstrated that extract D was devoid of any allergenic or antigenic activity, whereas extracts A and M were often regarded to be similar, if not identical. Although extracts S and L were demonstrated to be more potent by RAST inhibition, they failed to meet the specification requiring that the proposed IS be prepared from multiple *Alternaria*-source strains. The specifications for the proposed IS also required a minimum of 2 mg (dry weight) per ampule but did not specify a maximum dry weight fill. Extracts S and L contained approximately 20 mg per ampule (Table VB); therefore, when the test extracts were reconstituted with equal volumes of diluent, it was not surprising that extracts S and L assayed at a higher allergenic activity (Table III). If the allergenic potency figures are recalculated as specific activity (eg, RAST-inhibition activity per milligram of dry weight), extract M = A becomes the most potent sample tested. It should be emphasized that extracts S and L were each prepared from single-source strains that were also represented in extract A, the proposed IS. Overall, the RAST-inhibition results obtained in the collaborative study were very similar to the results obtained in the preliminary study.⁴ The relatively high GCV values noted in the RAST-inhibition assays (Table III) are due to the variety of solid-phase support systems and IgE antibody-containing sera used in the respective laboratories.

The variability noted in the CIE/CRIE results (Fig. 2; Table IV) was also the result of many factors, including the concentration of the test extracts, the quantity of rabbit antisera in the second dimension CIE gels, and the source of IgE antibodies and the incubation period used for the CRIE gels.

The variances observed in the protein assay results (Table VA) were more troublesome. These variances were, in part, due to the different assays used, and, in part, by the unreliability of color-based protein assays in complex, highly colored allergen extracts, such as molds; this unreliability has been noted by other investigators.²⁵ Although the results from amino acid analysis should be the most accurate, results from laboratories with this technique (Nos. 6 and 16) varied

by two- to threefold. This is perhaps due to the high carbohydrate concentration present in the *Alternaria* samples. Additional amino acid analyses will be performed with extract M = A in an attempt to resolve this question.

We have no definite explanation for the observation that the contents of some ampules of the proposed IS appeared shrunken after 1 year of storage. The problem appeared to be one of appearance only, as no differences in dry weight or allergenic activity per ampule were observed when ampules with shrunken contents were tested. A review of production records from the ATCC indicated that the precision of ampule filling was monitored by weighing every twentieth to five hundredth ampule; no filling errors could be identified from these records. We postulate that the somewhat higher than usual residual moisture in the proposed IS (2% to 4%) may have contributed to the change in appearance during the first few months of storage.

The data from this collaborative study have been submitted to the WHO, which will determine the acceptability of the extract as an IS. We suggest that an arbitrary international unitage of 100,000 per ampule be assigned. This extract should serve as a useful calibrator of other extracts in use by various researchers and clinicians. National allergen regulatory authorities may wish to compare existing national standards to the proposed IS.

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