

Biochemical and immunochemical methods for purification, characterization and standardization of allergen extracts

Roland Einarsson

Introduction

Allergenic extracts consist of complex mixtures of proteins with a considerable variation in allergenic activity and allergen composition. Therefore well-characterized and standardized extracts have been required by allergologists to increase the precision of diagnosis and the safety of specific immunotherapy. The allergens are protein molecules which also are immunogenic and consequently physico-chemical and immunochemical based methods are applicable for characterization and standardization. The purpose of the present paper is to focus on some important methods used in allergen research (quality control and standardization), their advantages and limitations.

Purification

Purification of allergenic extracts requires the use of suitable methods and by a combination of various methods (ultrafiltration, gel filtration, ion exchange chromatography, reversed phase chromatography etc.) purified allergen preparations are produced free from irrelevant constituents (small molecules, toxins, irritants etc.) but with retained allergenic activity (2). The manufacturing of allergenic extracts should be modelled after the production of pharmaceuticals and further controlled by applying various physicochemical and immunochemical methods. However, the quality of the final extract is directly dependent on the quality of the starting source material, its allergenic potency and allergenic composition. The analysis of allergenic extracts, source materials as well as purified material with respect to

quality control, standardization or stability include a number of biochemical and immunochemical methods in combination as no single method gives a satisfactory answer regarding the quality of the complex protein mixtures constituting most of the allergenic extracts.

Biochemical and electrophoretic characterization

Table 2 demonstrates some biochemical and electrophoretic methods in use for in process control of allergenic extracts as well as for quality control of the final preparations (2). The protein and carbohydrate content are easily measured biochemical properties suitable for checking the batch uniformity. The electrophoretic based techniques at constant pH or at a pH gradient in agarose or polyacrylamide as anticonvective media are most useful screening procedures for examining molecular properties of allergenic extracts (isoelectric points, molecular weights). As these techniques separate the allergenic protein components in distinct patterns, they might also be used for discrimination of extracts as well as for identification of clinically important allergens. The biochemical and electrophoretic methods have the advantage of requiring no immunologic or isotopic reagents. It is important however to be aware of the fact that these techniques only give limited information regarding the allergen content and potency.

Potency determination

The *in vitro* potency determinations of total allergenic activity are best performed

with the RAST-inhibition assay (7), where the capacity of the allergenic extract to inhibit the binding of specific IgE to the immobilized allergen is determined in relation to a well-characterized and standardized reference preparation (Fig 1). Another used laboratory method to evaluate allergenic extract potency is histamine release (4). When exposing leucocytes from allergic individuals to their specific allergens they will release histamine. Histamine release is more sensitive than RAST-inhibition but is rather complicated to perform and furthermore the requirement of freshly prepared leucocytes is another limiting factor. Therefore for routine use to measure total allergenic activity RAST-inhibition seems more suitable, but for studies of very diluted extracts histamine release is advantageous.

Crossed immunoelectrophoresis/ crossed radioimmuno-electrophoresis

Immunochemical methods using rabbit antibodies have been used for many years for the characterization and quantification of allergenic extracts. In Table 3 is demonstrated various methods based upon the use of monospecific antibodies for quantitative determination of individual allergens (1). The sensitivity of the methods can be further improved by incubation of the immunoprecipitates with patient-specific IgE, and ^{125}I -anti-IgE followed by autoradiography.

However, in the study of complex allergenic extracts information regarding all allergens as well as a rough quantitative estimate of major allergens is preferable. A powerful and more complex method for such an evaluation of allergenic extracts is crossed immunoelectrophoresis (CIE) and crossed radioimmuno-electrophoresis (CRIE) (3,6) (Fig 2). CIE/CRIE and some modifications of them are applicable methods for characterization of allergens and also for evaluating the patient's reactivity against individual allergen. Interpretation of CIE/CRIE data is based upon the assumption that the immunized rabbits have responded with precipitating antibody to all allergens. Quantitative immunoelectrophoretic methods have been widely used for the quality control of allergenic extracts (al-

lergenic composition, recovery during manufacturing, standardization, stability, identification of clinically important allergens) (2,3). Furthermore these methods are well correlated to the *in vitro* assay of allergenic potency by means of RAST-inhibition.

Nitrocellulose immunoprint

Nitrocellulose immunoprint (protein-gel-blot) is another gel-based method for studying individual allergens in complex allergenic extracts and for screening of patient sera for specific IgE antibodies (5). The separated proteins are immobilized on nitrocellulose, detected by incubation with patient serum and labelled anti-IgE followed by autoradiography or zymography (Fig 3). This technique is rapid and high-resolving and in contrast to CIE/CRIE is not dependent on rabbit antibodies, which might be a limiting factor for the CRIE procedure. CRIE as well as immunoprint can also be used to study the binding of patient's specific IgG antibodies to individual allergens by using labelled anti-IgG instead of labelled anti-IgE. This latter approach is especially interesting when applying the immunochemical methods for monitoring patients during immunotherapy.

REFERENCES

1. Axelsen, N.H. (Ed.): Handbook of Immunoprecipitation in Geltechnique. Scand. J. Immunol. 17, Suppl. 10, (1983).
2. Dreborg, S., Einarsson, R. and Longbottom, J.: The chemistry and standardization of allergens. In Handbook of Experimental Immunology (D.M. Weir, C.C. Blackwell and L.A. Herzenberg eds.) 4th Edition, (1985) (in press).
3. Löwenstein, H.: Quantitative immunoelectrophoretic methods as a tool for the analysis and isolation of allergens. Progress in Allergy, 25, 1—62, (1978).
4. Siraganian, R.P.: An automated continuous-flow system for the extraction and fluorometric analysis of histamine. Anal. Biochem. 57, 383—394, (1974).
5. Towbin, H. and Gordon, J.: Immunoblotting and Dot Immunobinding — Current status and outlook. J. Immunol. Methods 72, 313—340, (1984).
6. Uhlin, T. and Einarsson, R.: A rapid procedure for crossed radioimmuno-electrophoresis. Int. Archs Allergy appl. Immunol. 70, 213—219, (1983).
7. Yman, L., Ponterius, G. and Brandt, R.: RAST-based allergen assay methods. Dev. Biol. Standard. Vol. 29, 151—165, (1975).

Table 1
Chromatographic methods used in allergen research

Method	Application
Gelfiltration	Separation of molecules based upon size
Ion exchange chromatography	Separation of molecules in relation to charges
Reversed phase chromatography	Separation of molecules according to polarity

Table 2
Biochemical and electrophoretic methods used in allergen research.

Method	Application
Lowry method	Determination of total protein content
Amino acid analysis	Determination of individual amino acids; total protein content
Anthrone method	Determination of hexoses (carbohydrates)
Gas liquid chromatography	Quantification of individual monosaccharides; total carbohydrate content
Gradient Polyacrylamide gel electrophoresis	Molecular weight determination in native gradient
SDS-gradient Polyacrylamide gel electrophoresis	Molecular weight determination in solubilizing media
Isoelectric focusing	Protein composition (stained bands)

Table 3
Immunological methods used in allergen research

Method	Application
Single radial immunodiffusion	Quantitation of individual antigens; monospecific antiserum
Rocketimmunoelectrophoresis	Quantitation of individual antigens; monospecific antiserum
Radio rocket immunoelectrophoresis	Identification and quantitation of allergens
CIE	Characterization of allergen extracts with respect to antigenic composition
Tandem-CIE	Comparison of antigens (partial identity)
CLIE	Comparison of antigens (partial identity)
CRIE	Identification of IgE and IgG antibodies in patient sera; identification of allergens in extracts
Nitrocellulose immunoprint	Characterization of allergen extracts; identification of IgE and IgG antibodies in patient sera

Fig 1.

Potency determination of a birch extract (a) in relation to a birch reference preparation (b) with RAST-inhibition. Discs carrying the birch extract and using a patient serum pool.

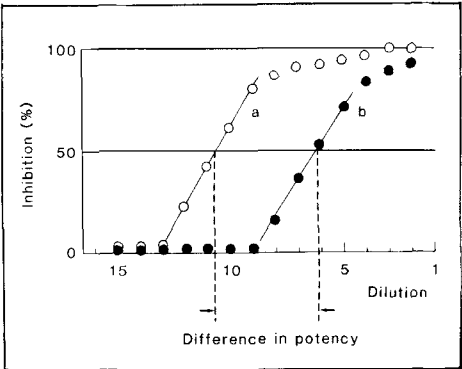
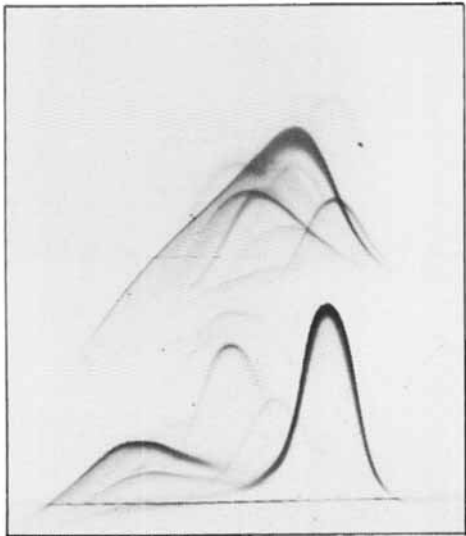


Fig 2.

a) Crossed immunoelectrophoresis of Poodle/Alsation dandruff extract (50/50 w/w) against hyperimmune rabbit anti-sera after protein staining with Coomassie Brilliant Blue.



b) Corresponding x-ray film from autoradiography experiment of the same plate. The radiostaining pattern is exposed to correspond to CRIE class G.

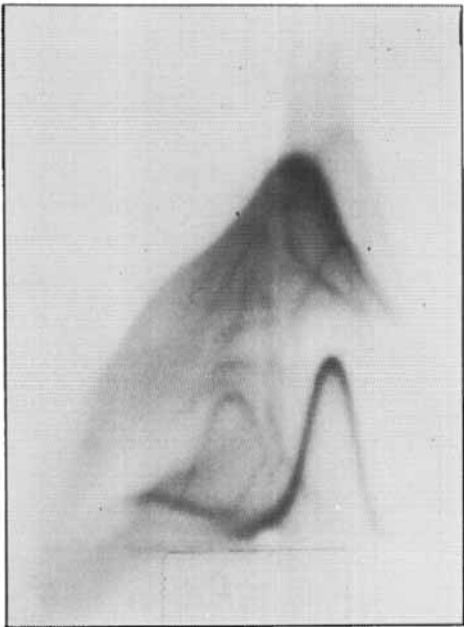
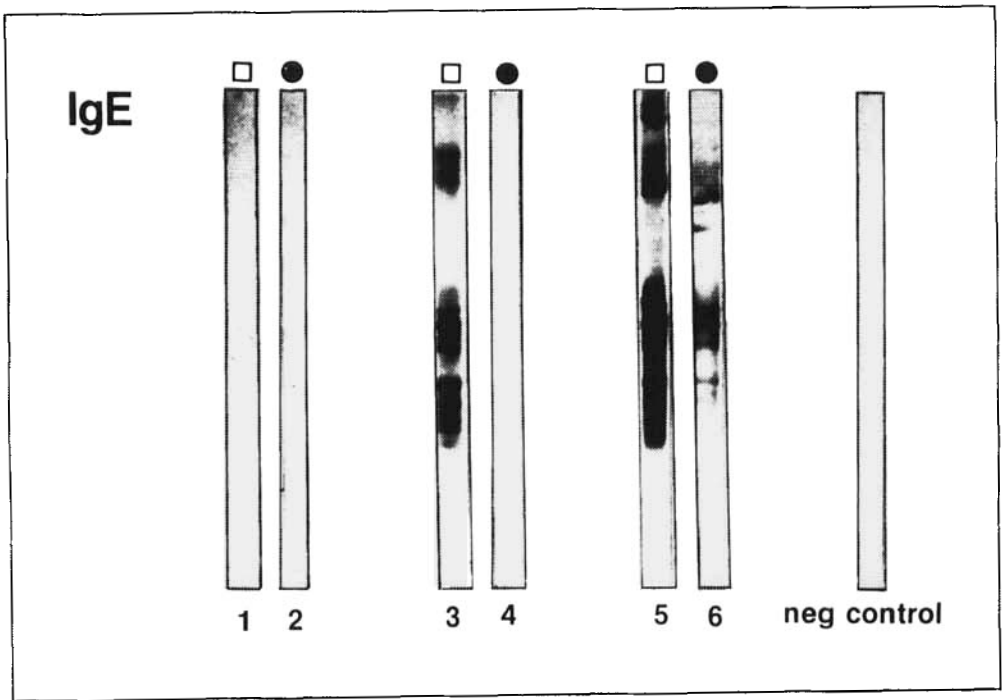


Fig 3.

Binding of patient specific IgE antibodies to Short Ragweed (*Ambrosia elatior*) proteins (autoradiographic detection, exposure time 15 hours). Nitrocellulose

strips 1 and 2, 3 and 4, 5 and 6 represent three different patients before and after treatment with Short Ragweed extracts. The blank strip to the right is a negative control.

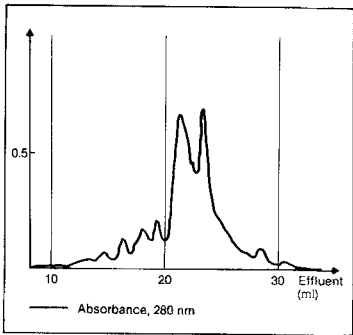


PRINCIPLE: GEL FILTRATION SIZE

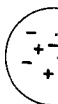


- Proteins penetrate the gel beads to different degrees
- Large proteins eluted first
- Small proteins eluted late

YELLOW JACKET

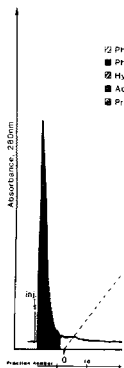


PRINCIPLE: ION EXCHANGE



- Proteins adsorb to different degrees
- Proteins are eluted at different pH

YELLOW JACKET



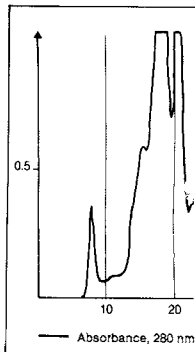
Purification of Allergens

APPLICATION:

ALLERGEN
EXTRACT

GEL FILTRATION SUPEROSE 12

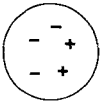
- Gel filtration of birch pollen extract on Superose 12 is used for partial purification of the allergens



0.05 M 1.3 dia
pH 8.5 0.5 ml/min

AIM: Separation for biological and chemical analysis of individual allergens

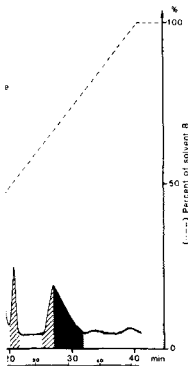
CHANGE CHARGE



to the ion

ed at different
f salt or at
gradient

JACKET

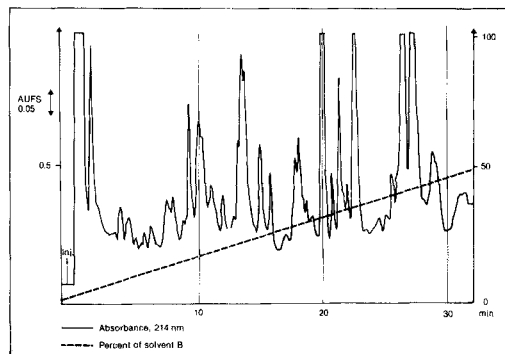


PRINCIPLE: REVERSED PHASE POLARITY

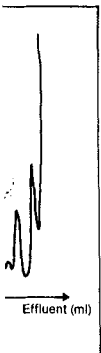


- Proteins adsorb by interaction between nonpolar groups on the protein and in the gel
- Proteins are eluted at different concentration of a nonpolar solvent in a gradient

YELLOW JACKET



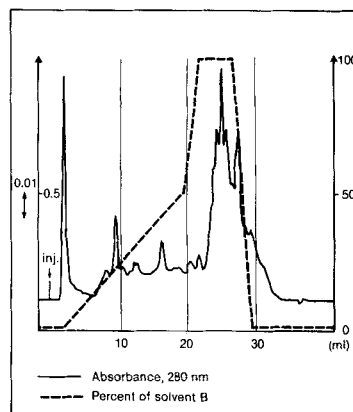
irch allergens



propane

ION EXCHANGE CHROMATOGRAPHY MONO Q

- Separations are done at a pH where the allergens adsorb to Mono Q and where contaminating proteins differ in charge density



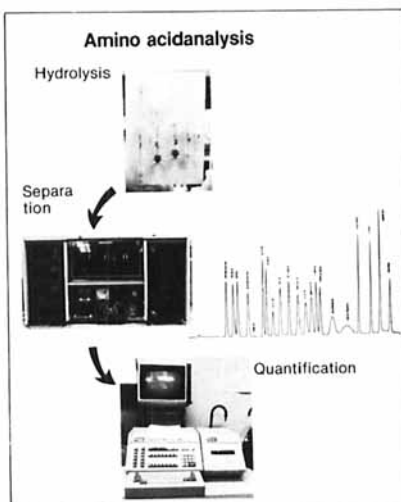
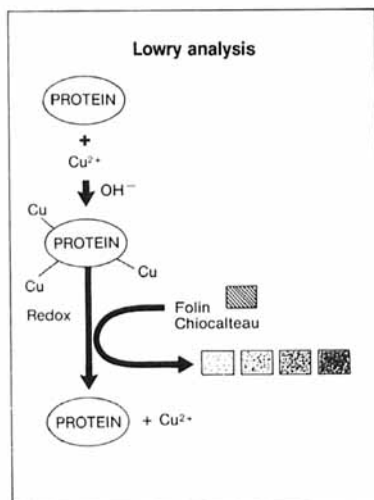
0.01 M 1.3 diaminopropane pH 0.9;
0—0.5 M Na-acetate 1 ml/min

ELECTROPHORESIS

Anne Annerhed, Bo Renck

PROTEIN ASSAYS

AIM: Determination of protein content



+ Simple
cheap

— Depending on standard
sensitive for interference

+ Specific
Expensive

— Complex equipment

Mites			
Dermatophagoides			
Pteronyssinus		Farinae	
L	A	L	A
17,4	36,7	29,5	39,1
16,1	38,6	29,1	42,2
20,4	37,5	33,1	41,3
19,6	38,7	29,5	46,2
19,0	34,1	23,7	39,3
18,5	37,1	—	29,0
1,735	1,879	S	3,369
9,38	5,07	%CV	11,62

Moulds			
Alternaria Alternata		Cladosporium Herbarium	
L	A	L	A
2,4	13,6	8,3	26,6
1,8	13,4	12,2	27,6
4,8	13,0	7,8	26,7
8,4	13,5	7,3	28,7
2,5	13,7	7,7	23,6
4,0	13,4	—	8,7
2,723	0,270	S	2,011
68,06	2,01	%CV	23,22

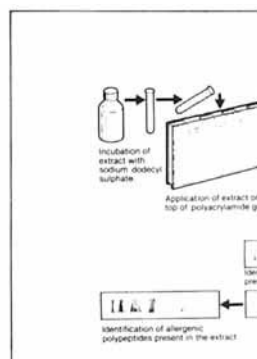
Epithelium			
Dog		Cat	
L	A	L	A
29,2	46,1	49,9	56,0
32,7	44,8	59,2	51,0
19,5	46,8	55,8	62,3
18,9	45,3	48,0	64,0
23,9	45,6	52,0	58,9
24,8	45,7	—	53,0
6,031	0,766	S	4,525
24,32	1,68	%CV	8,34

Pollen								
Species	Grass		Species	Trees		Species	Weeds	
	L	A		L	A		L	A
g3	39,8	50,2	t1	48,4	51,6	w6	15,0	25,1
g5	46,3	46,3	t2	58,8	51,9	w9	24,4	39,3
g6	50,7	47,0	t3	43,6	50,2	w10	45,4	38,3
g8	45,4	56,9	t4	39,9	41,2			
g13	44,4	41,0	t7	32,6	20,4			

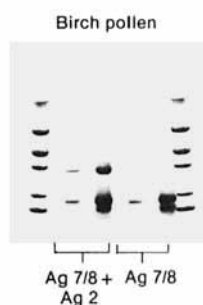
SDS — GRADIENT

AIM: Determination of

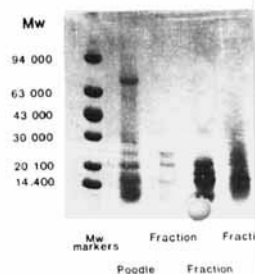
Principle



Applications

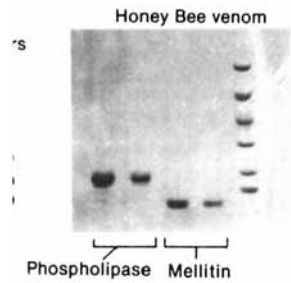
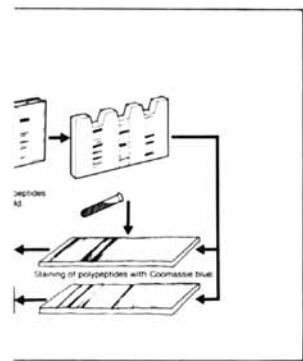


SDS-PAGE

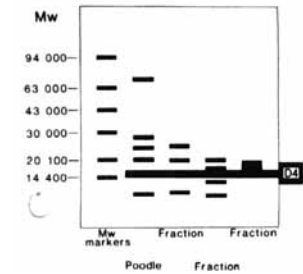


ELECTROPHORESIS

ular weight (Mw)



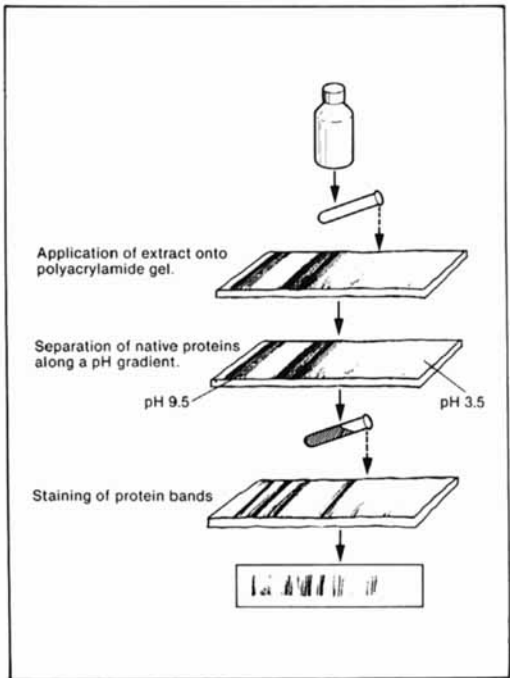
der
DIENT GEL 4/30



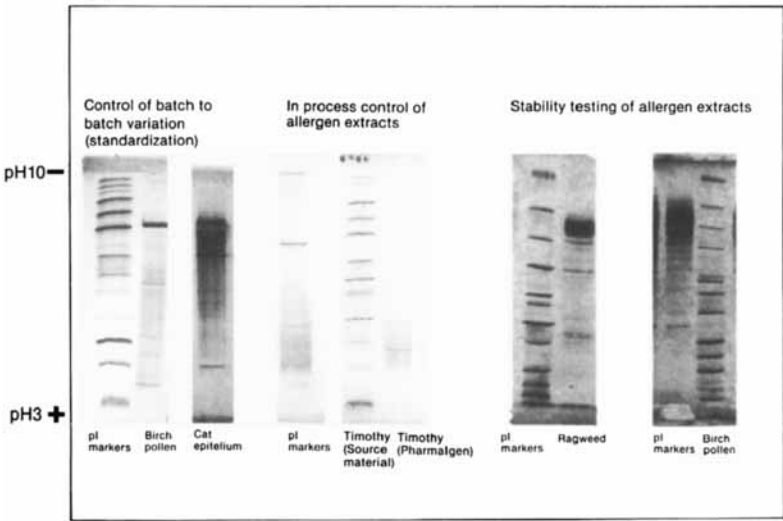
ISOELECTRIC FOCUSING

AIM: Characterization of allergen extracts
in pH gradients

Principle



Applications



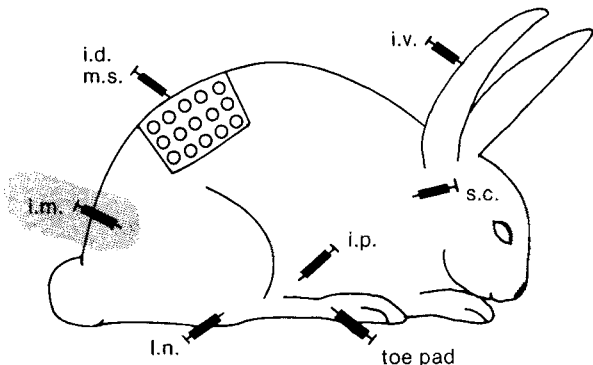
CROSSED IMMUNOELECTROPHORESIS

CROSSED RADIO IMMUNOELECTROPHO

PRODUCTION OF ANTISERUM

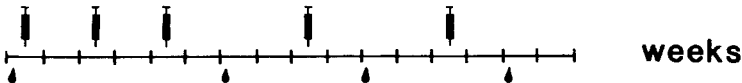
AIM: Produce a well defined antiserum

- Standardized immunization procedure
 - the same administration site

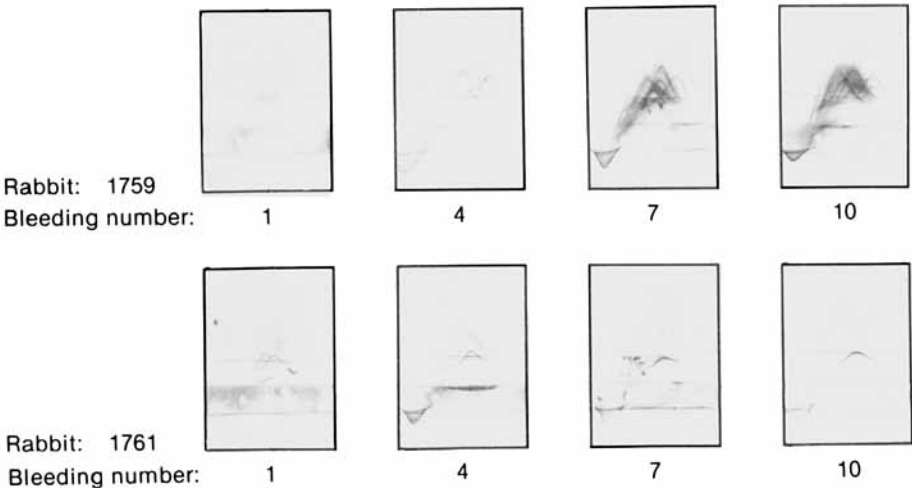


- the same immunization schedule

(Antigen dose 100-500 μ g,
Freund's adjuvant, i.m.)



2) pool of antiserum



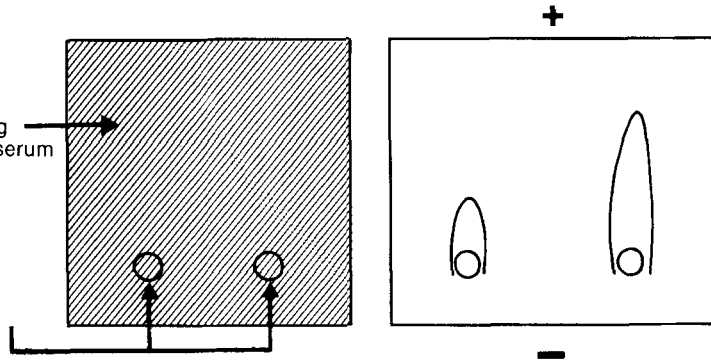
ROCKET IMMUNOELECTROPHORESIS

AIM: Quantitation of individual antigen

PRINCIPLE:

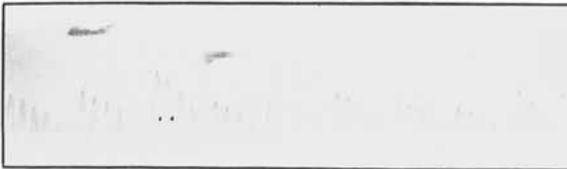
Agarose containing monospecific antiserum

Antigen sample

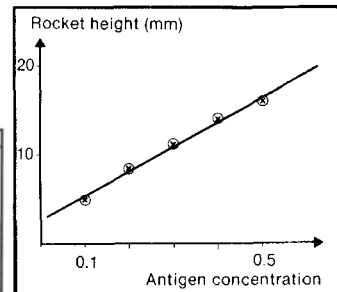


APPLICATION

Content of allergen P1 in different batches of d1 mite (*D. pteronyssinus*)

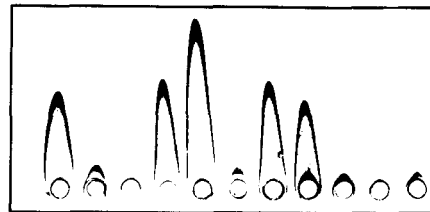


Calibration curve of P1



Content of Ag8* in different strains of *Alternaria alternata*

* Ag8 is partially identical to Alt. 1.



CROSSED IMMUNOELECTROPHORESIS CROSSED RADIO IMMUNOELECTROPHORESIS

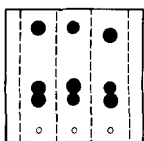
CROSSED RADIO IMMUNOELECTROPHORESIS (CRIE)

AIM: Evaluation of antigenic/allergenic composition of allergenic preparation

PRINCIPLE:

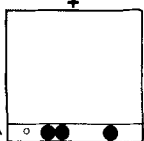
CROSSED IMMUNOELECTROPHORESIS (CIE)

First dimension



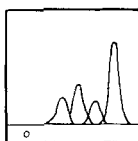
Electrophoretic separation of protein mixture in agarose gel

Second dimension



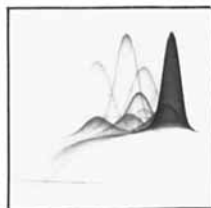
Electrophoresis of proteins (antigens) into antibody-containing agarose gel

Staining

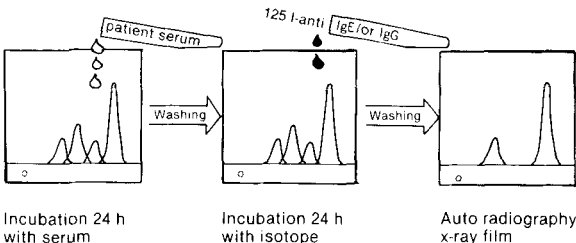


The formed immuno precipitates are protein stained

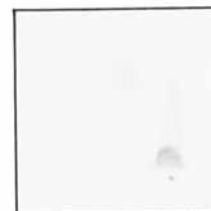
CIE Cat



CROSSED RADIO IMMUNOELECTROPHORESIS (CRIE)



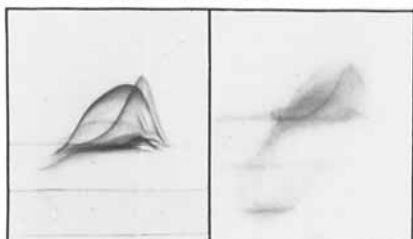
CRIE Cat



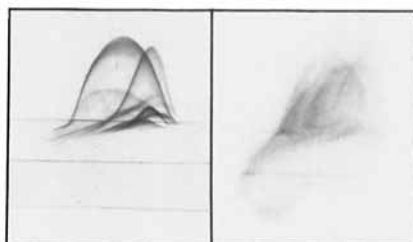
IE)

APPLICATIONS:
CONTROL OF ALLERGEN COMPOSITION.

Source material mite d2

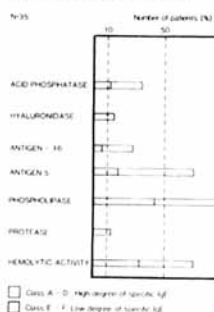


Pharmalgen mite d2

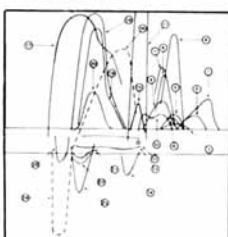


CRIE CLASSIFICATION (A-G) OF ALLERGENS (YELLOW JACKET)

FREQUENCY TABLE



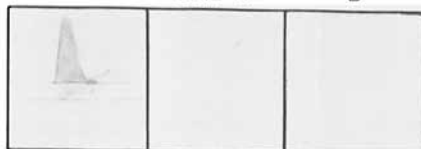
REFERENCE



CIE

CRIE A

B



C

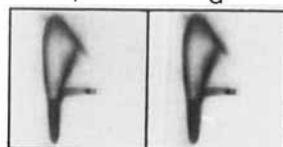
D

E



F

G



MONITORING IMMUNOTHERAPY

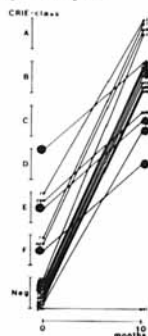
(*Cladosporium herbarum*)

Gladosporium herbarum
immunotherapy

Patient No.	Top dose BU
5	60 000
9	100 000
21	8000
22	800
26	4000
31	3000
41	10 000
2	100 000
3	60 000
7	100 000
13	60 000
25	45 000
29	4000
42	50 000
46	100 000
47	50 000

● Patient with IgE towards antigen 12
 ○ Patients with no detectable IgE towards antigen 12

lgG-CFIE Antigen 12

[illegible]

Negative control serum

Upper: Before treatment Change: + IgG-EMV

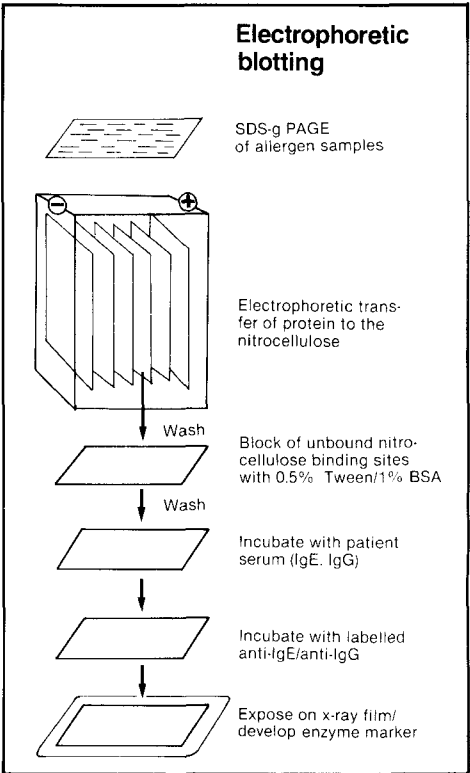
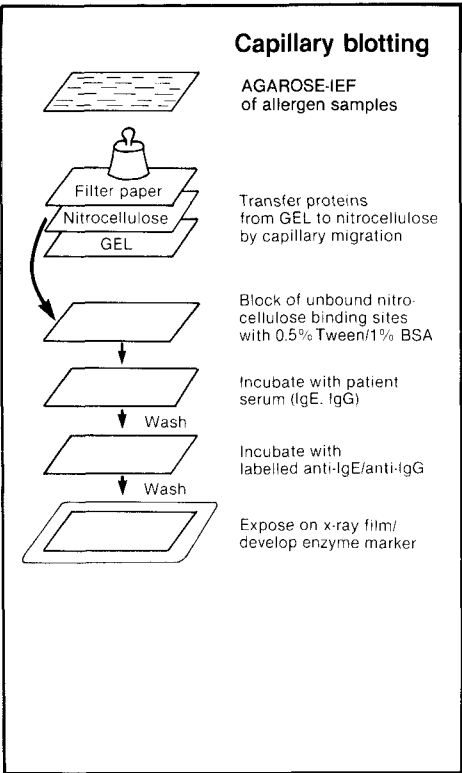
Lower: 10 months treatment

IMMUNOPRINT

Björn Persson and Ingrid Steringer

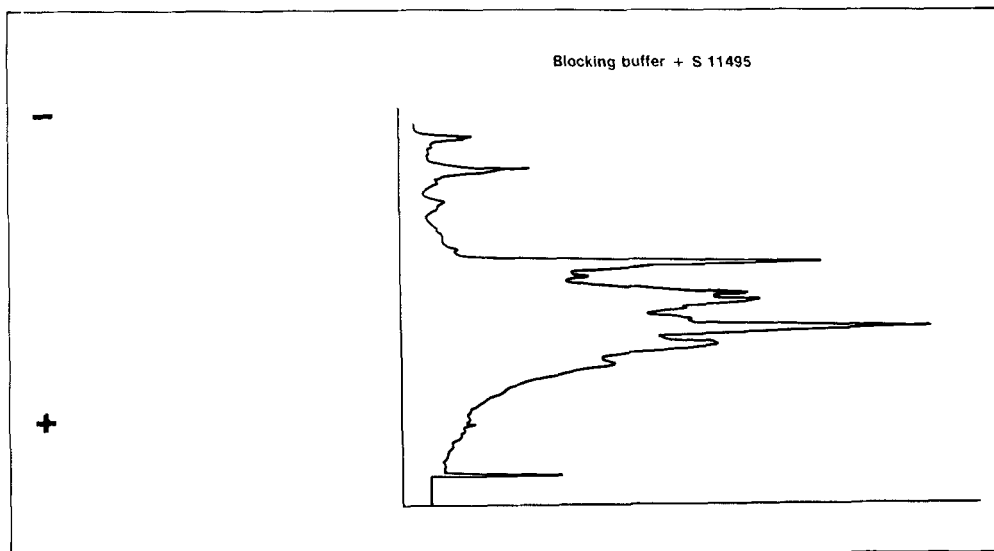
AIM: A method based upon combination with electrophoresis for the detection of allergenic compounds

PRINCIPLE: The method is a combination of a high resolving separation technique with a convenient solid phase assay



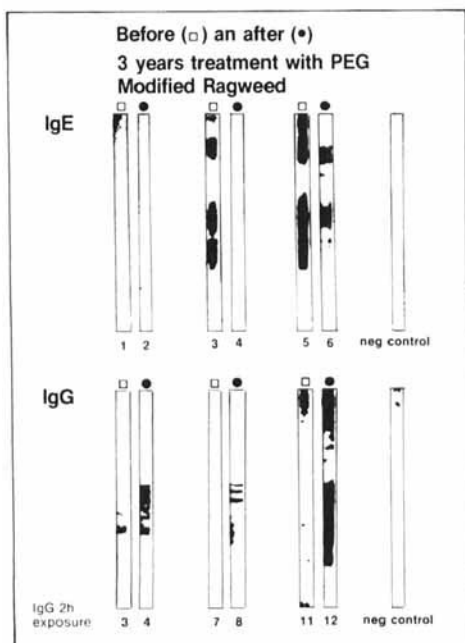
APPLICATIONS

Tree-mix allergen separated by IEF, followed by capillary blotting and isotope detection



Monitoring of patients during immunotherapy

Allergens separated by IEF and transferred to nitrocellulose by capillary blotting



Patient serum tested for three different allergens

Allergens separated by SDS-g PAGE and transferred to nitrocellulose by electro-blotting followed by isotope detection.

