

CHROMSYMP. 1381

ANALYSIS OF ALLERGENIC COMPONENTS OF A *PARIETARIA JUDAICA* POLLEN EXTRACT BY CHROMATOGRAPHIC METHODS FOR THE EVALUATION OF PURIFICATION PROCEDURES

ANGELA BASSOLI, FRANCESCO CHIOCCARA, GIUSEPPINA DI GREGORIO, BRUNO RINDONE* and STEFANO TOLLARI

Dipartimento di Chimica Organica e Industriale, Universita' di Milano, Via Venezian 21, 20133 Milan (Italy)

PAOLO FALAGIANI and GRAZIELLA RIVA

Lofarma Allergeni, Reparto Ricerche, Viale Cassala 40, Milan (Italy)

and

EZIO BOLZACCHINI

Istituto Scientifico H. S. Raffaele, Via Olgettina 60, 20132 Milan (Italy)

SUMMARY

Size-exclusion and ion-exchange high-performance liquid chromatography were used to monitor the presence of polymeric aggregates and of families of allergens of similar isoelectric point in *Parietaria judaica* pollen extracts. A radio-allergo-sorbent test and chromatofocusing were used for the analysis of individual fractions. This allows the detection of two groups of allergens, having *pI* of 4.7 and > 5.7, respectively, and shows the effect of dialysis or ultrafiltration on the distribution of allergens.

INTRODUCTION

Since *Parietaria* (*Parietaria judaica*) allergenic extracts are used therapeutically in the Mediterranean area, standardization of the extracts and studies of their chemical composition are needed.

We have attempted to purify *Parietaria* pollen extracts with the use of size-exclusion high-performance liquid chromatography (SEC-HPLC)¹ or of a combination of SEC-HPLC and ion-exchange HPLC (IEC-HPLC)², and other groups have used SEC-HPLC³, gel chromatography⁴, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)⁵, immunochemical methods⁶ or crossed radioimmuno-electrophoresis (CRIE)^{7,8}.

Since an improvement in the purification resulted from combinations of techniques both for *Parietaria*, where SEC-HPLC and IEC-HPLC were used², and with timothy (*Phleum pratense*), where gel chromatography and IEC⁹ or IEC-HPLC and SEC¹⁰ were used, in a recent paper¹¹ we suggested a new sequence of enrichment steps constituted by ultrafiltration and IEC-HPLC. This method was further developed on a micropreparative scale¹².

The results obtained from the chromatographic purification experiments^{1-3,11,12} seemed to be different from those obtained from immunochemical purification experiments⁴⁻⁸. This suggested that the purification technique modified the composition of the allergenic mixture originally present in a *Parietaria* pollen extract. Hence, a clarification of this point was important for the standardization of *Parietaria* pollen extracts to be used in therapy.

In this paper we report an attempt to characterize different families of allergens in this pollen extract and to study their behaviour in the extraction procedure and purification techniques.

MATERIALS AND METHODS

Parietaria pollen extract

A 10-g sample of dry pollen obtained from Allergon (Engelholm, Sweden) was extracted with 200 ml of 0.15 M phosphate buffer (pH 7.2) for 24 h at 4°C. The pollen grains were filtered and the extract was dialysed for 48 h against water, using a Spectra/Por 3 membrane (Spectrum Medical Industries, Los Angeles, CA, U.S.A.) with a molecular weight cut-off of 3500, then filtered through a 0.22- μ m membrane (Millipore, Bedford, MA, U.S.A.), and lyophilized.

The same extraction procedure was used at 20°C in experiments with different extraction times.

HPLC analyses

HPLC size-exclusion analyses were performed by dissolving lyophilized pollen extracts in 0.15 M phosphate buffer pH (6.8), 0.5 M potassium chloride (protein content 2 mg/ml) and injecting through a Rheodyne 10- μ l loop (60 μ g per injection). The instrument was a 5000 HPLC isocratic system (Varian, Palo Alto, CA, U.S.A.), equipped with a MicroPak TSK 2000 SW (30 cm \times 1.8 in. O.D. \times 8 mm I.D.) size-exclusion column and a TSK SW guard column (7.5 cm \times 10 mm O.D. \times 8 mm I.D.) (Tokyo Soda, Tokyo, Japan), eluted with 0.15 M phosphate buffer (pH 6.8), 0.5 M potassium chloride at a flow-rate of 1 ml/min. The detector was a Varian UV 50 instrument set at 280 nm.

Ion-exchange HPLC with an ionic strength gradient was performed by dissolving the material in 10 mM Tris-acetic acid buffer (pH 7.0)-20 mM sodium acetate and injecting through a Rheodyne 10- μ l loop. A Varian 5500 HPLC instrument equipped with a TSK DEAE-5PW ion-exchange column (7.5 cm \times 1.8 in. O.D. \times 7.5 mm I.D.), was employed. Gradient elution was performed with eluents A, a mixture of 10 mM Tris-acetic acid buffer containing 20 mM sodium acetate and B, 10 mM Tris-acetic acid buffer containing 500 mM sodium acetate, at a flow-rate of 1 ml/min.

Chromatofocusing

Chromatofocusing was performed by dissolving the material in 25 mM Bis-Tris (pH 6.00) (1 M hydrochloric acid) and eluting with Polybuffer 74 (Pharmacia, Uppsala, Sweden), diluted 1:7 (v/v), and 4% taurine (pH 4.00) (1 M hydrochloric acid). The instrument was a fast protein liquid chromatograph (Pharmacia, Uppsala, Sweden) equipped with an LCC-500 controller, two P-500 pumps and two V-7 valves,

a flow-through pH electrode and a single-path variable-wavelength detector (Waters Assoc., Milford, MA, U.S.A.). The volume of the injection loop was 50 μ l. A pre-packed ion-exchange column (Mono PTM, HR5/20,200 mm \times 5 mm I.D.; Pharmacia) was jacketed and the temperature of 25°C was maintained by use of an external water-circulation bath. Fractions of 2 ml were collected, dialysed and subjected to radio-allergo-sorbent test inhibition (RAST-I).

Radio-allergo-sorbent test (RAST) of the chromatographic fractions

The fractions (75 μ l each) were bound to cyanogen bromide-activated paper¹³ discs, and direct RAST or RAST-I was performed according to Yman *et al.*¹⁴, using a pool of sera from 95 patients with high sensitivity to *Parietaria judaica* pollen.

RESULTS AND DISCUSSION

The influence of the extraction time on the composition of *Parietaria* pollen extracts was tested by performing extraction experiments with 0.5-, 60- and 720-min exposure of the pollen to the extracting buffer at 20°C and monitoring the results by SEC-HPLC and RAST. These experiments were performed either on the crude extraction solution or after dialysis of the extracts, thus allowing the influence of these molecular-weight-based refinement procedures to be demonstrated.

Fig. 1d shows the SEC-HPLC result of a 0.5-min extract which had undergone dialysis with a cut-off at 3500 daltons and its RAST analysis. The unique peak which appeared was centered at the molecular weight of 12 500 daltons previously reported¹⁰, and could correspond to the component isolated by another group with the same technique³, and allergenic activity was present as shown in the figure. The analysis of the corresponding non-dialysed material is shown in Fig. 1a. Some lower-molecular-weight components with some degree of allergenic activity were present in this case, indicating that material of molecular weight < 6500 daltons reacts also with the antibodies contained in the pool of sera used for the RAST test. They could, in principle, be fragments of the original allergenic proteins that still contain the allergenic determinant, or a distinct group of allergens. The chromatographic profiles of the 720, 60- and 0.5-min extracts non-dialysed were different, and their RAST profiles were also different (Fig. 1a, b, c). Dialysis of these samples gave chromatographic and RAST profiles similar to that shown in Fig. 1d, irrespective of the extraction time. A large amount of crude extract was subjected to two ultrafiltration procedures, with molecular-weight cut-offs of 100 000 and 10 000 daltons. The SEC-HPLC analysis of this ultrafiltrate showed a chromatographic and RAST profile similar to that shown in Fig. 1d. It can be concluded that dialysis and ultrafiltration equalize samples of *Parietaria* pollen extract for further treatments, but give an allergen distribution different from that of the original material, probably suppressing or altering some families of allergenic components.

The SEC-HPLC technique also allowed us to monitor the fate of the exclusion peak (MW > 150 000 daltons) noted in preparations of the allergenic extract when the dialysed and ultrafiltered extract was stored in a refrigerator at 4°C for a long time (Fig. 2). This disappeared when a mobile phase containing 2 or 4% urea was used, suggesting that the exclusion peak is a polymer of the protein material, centered around MW 12 500 daltons. Urea acts in glycoprotein chemistry as a dissociating

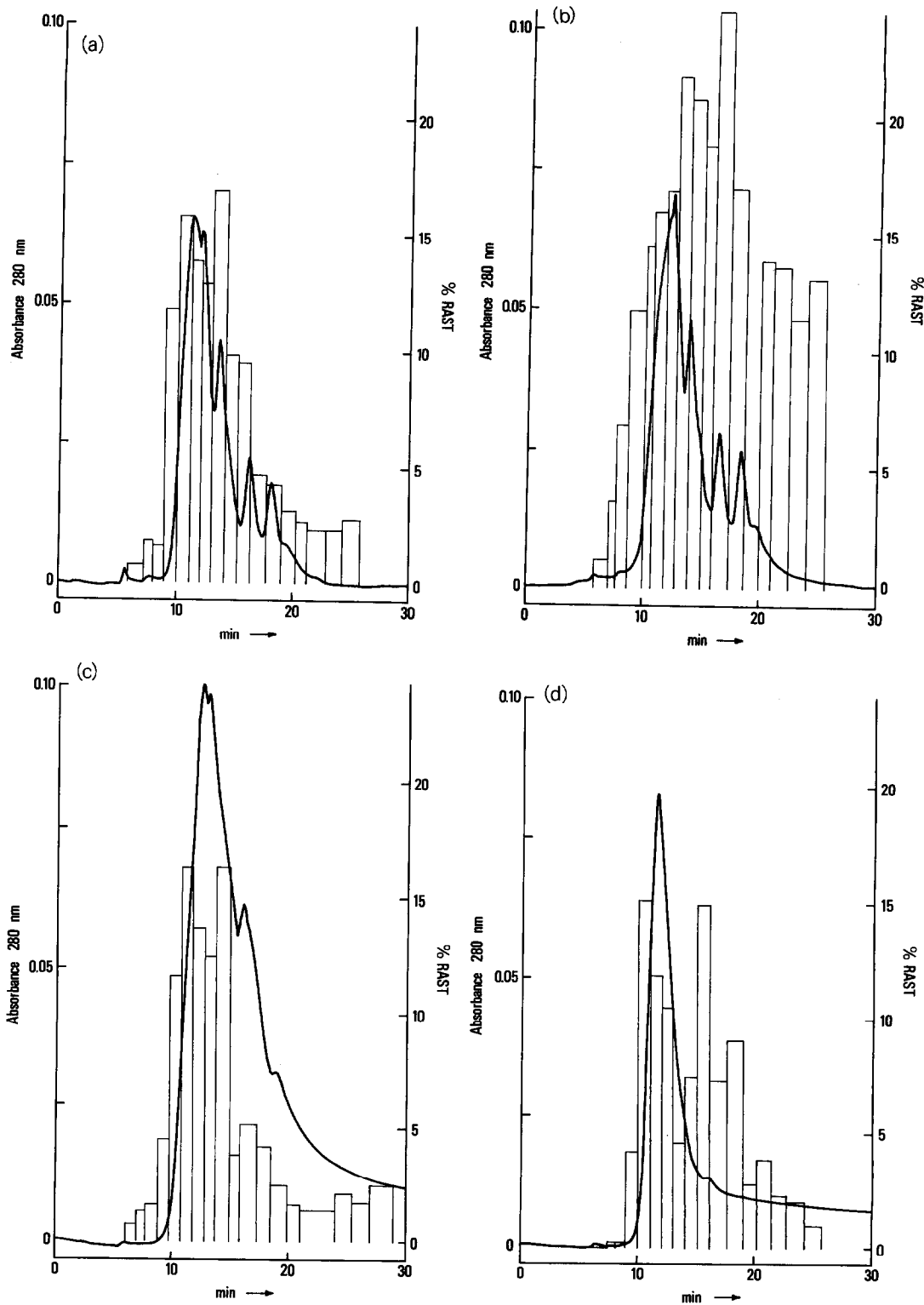


Fig. 1. Size-exclusion HPLC profiles of *Parietaria* pollen, extracted for 0.5 (a), 60 (b) and 720 min (c) and RAST analysis of the fractions. (d) SEC-HPLC profile of *Parietaria* pollen extracted for 0.5 min, dialysed and RAST analysis of the fractions.

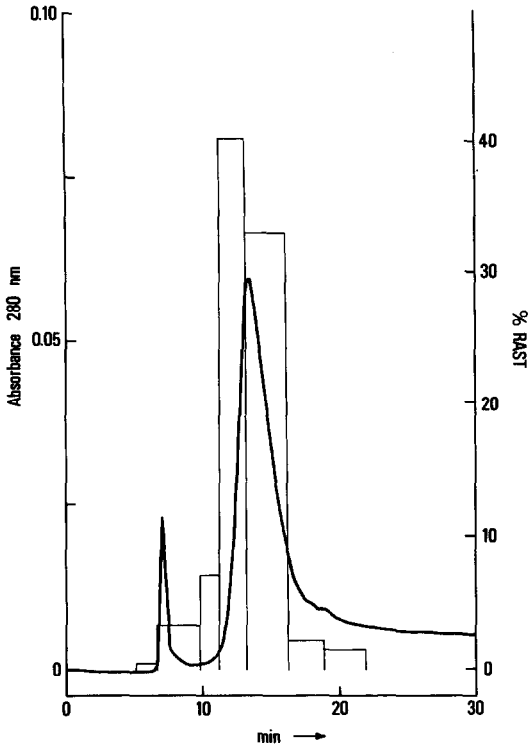


Fig. 2. SEC-HPLC profile of *Parietaria* pollen extracted for 24 h, and RAST analysis of the fractions.

agent of supramolecular aggregates¹⁵, held together by hydrophobic interactions. Confirmation of the nature of the exclusion peak in some *Parietaria* pollen extracts was obtained by collecting the SEC-HPLC fraction centered at MW 12 500 daltons and reinjecting in the same column: aggregates giving the exclusion peak were again present. Hence, a monomer-polymer equilibrium appears to exist and could be the result of storage in solution of the ultrafiltered and dialysed material in the absence of dissociating solutes, such as urea, SDS, etc. A further confirmation of this equilibrium came from the fact that there was RAST activity in the exclusion peak, suggesting a release of the allergens recognized by the RAST system under the conditions of the test. It can be concluded that the elimination of the exclusion peak from the pollen extracts results in a loss of allergenic material and should be avoided.

IEC-HPLC could be useful to define some characteristics of the population of allergens in the crude extract and of the allergens that were modified or eliminated from the extract by the dialysis or the ultrafiltration procedure. The IEC-HPLC chromatographic profiles of the samples obtained after different extraction times were different. The relative amounts of allergens in the first part of the chromatogram, where basic or neutral components having high RAST-I activity appear, increased with the extraction time (Fig. 3a-c). However, many of these allergens were eliminated by dialysis (Fig. 4a-c), and the same result was obtained in the ultrafiltration

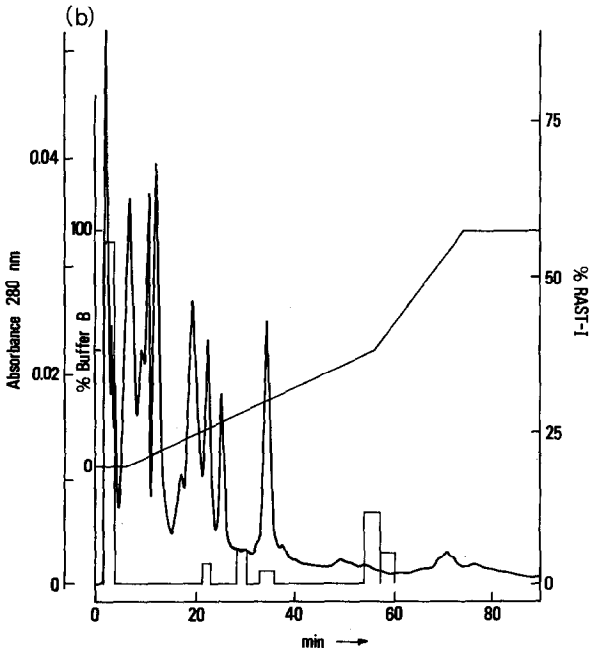
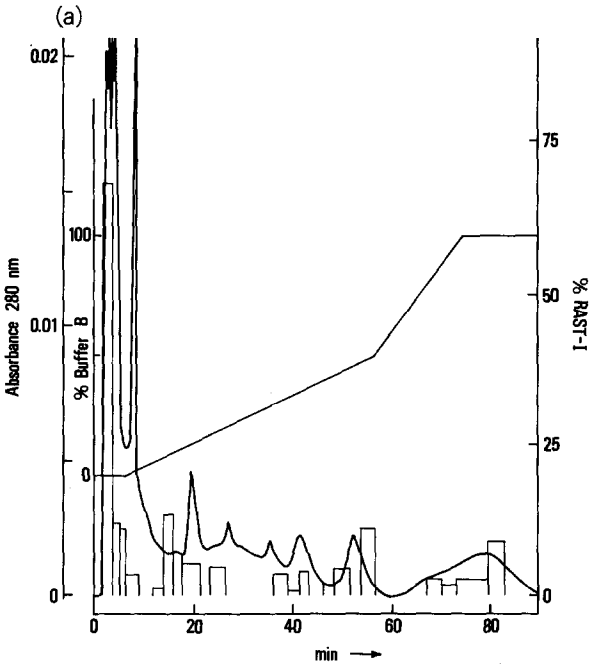


Fig. 3.

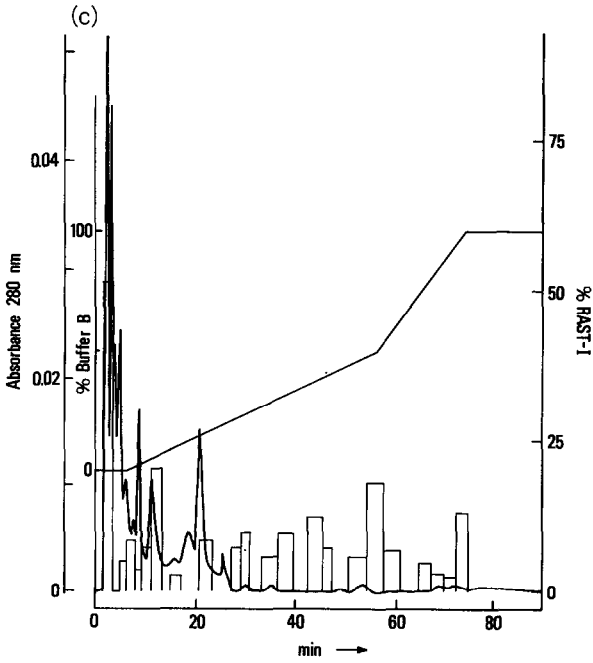


Fig. 3. Ion-exchange HPLC profiles of Parietaria pollen extracted for 0.5 (a), 60 (b) and 720 min (c), and RAST-I analysis of the fractions.

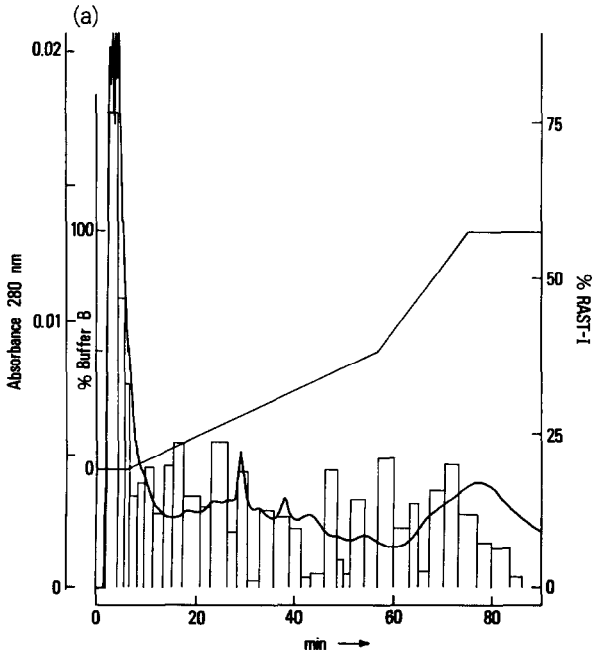


Fig. 4.

(Continued on p. 216)

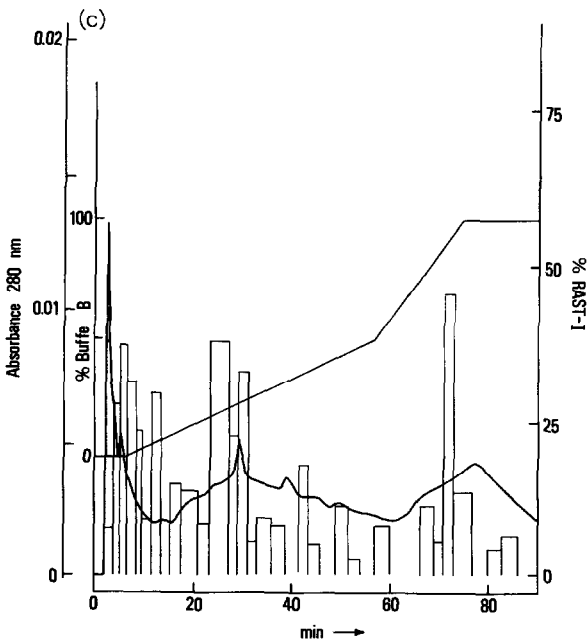
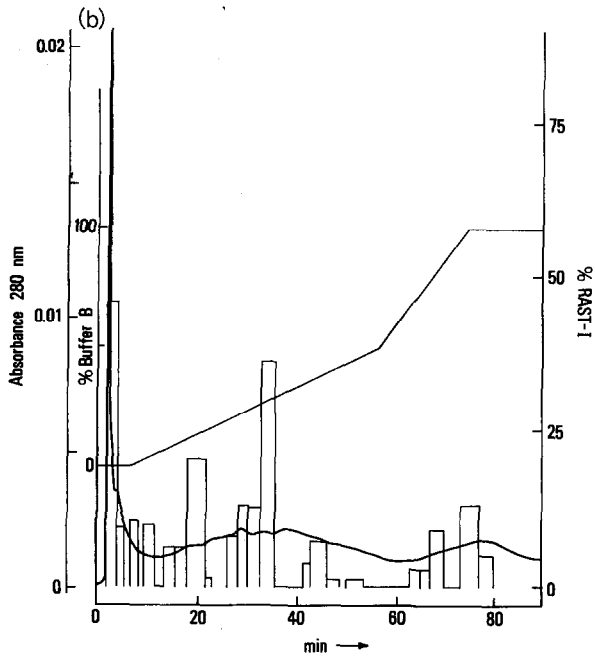


Fig. 4. Ion-exchange HPLC profiles of *Parietaria* pollen extracted for 0.5 (a) 60 (b) and 720 min (c), dialysed and RAST-I analysis of the fractions.

procedure. Thus, the equalization of the extract due to dialysis or ultrafiltration, noted in SEC-HPLC, was apparent also in IEC-HPLC.

The combination of the results of the experiments performed by SEC-HPLC and IEC-HPLC leads to the conclusion that two groups of allergens are present in *Parietaria* pollen extract: a first group of basic or neutral components, and a second group of acidic components. A chromatofocusing experiment confirmed this. A sample of high biological activity from an IEC-HPLC micropreparative experiment¹² was injected in a chromatofocusing column and gave the results shown in Fig. 5. Here, together with a RAST-I activity centered at pI 4.7, a RAST-I activity at $pI > 5.7$ is also present. The former group of allergens had been identified^{6,11}, whereas only one basic allergen is known at the moment: that from *Phleum pratense*, which has pI 9.30¹⁰.

Inspection of IEC-HPLC chromatograms and RAST-I analysis of the samples obtained after different extraction times shows the important modifications due to dialysis of the material. RAST-I activity was concentrated mainly in the first part of the chromatogram in the 0.5-min extraction procedure. Hence, basic allergens were initially extracted. Enrichment in RAST-I activity throughout the chromatogram appeared at longer extraction times, suggesting that under these conditions acidic allergens were also extracted. Equalization of the samples occurred after dialysis, and RAST-I activity was found in all the regions of the chromatogram. This suggests that the group of basic allergens responsible for the most part of the RAST-I activity in the 0.5-min extract are transformed under the conditions of dialysis. This leads to

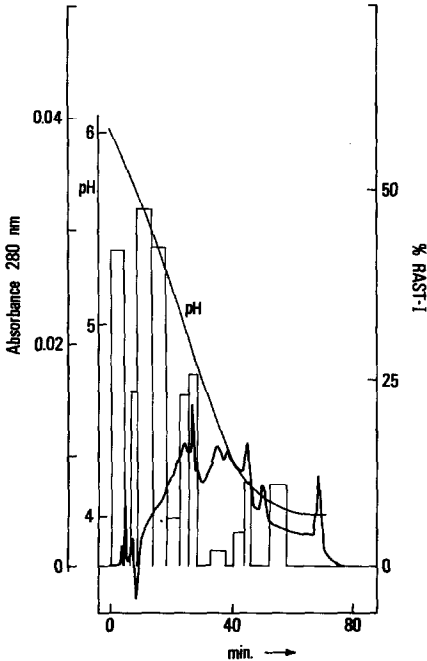


Fig. 5. Chromatofocusing profile of a fraction of IEC-HPLC enriched *Parietaria* pollen extract and RAST-I analysis of the fractions.

the conclusion that dialysis of the crude extract may alter the original composition in allergens.

In conclusion, work-up of *Parietaria* pollen extracts may delete some families of allergens. The combination of SEC-HPLC and IEC-HPLC with RAST-I may help in determining the type of allergens in these extracts and could be useful in standardization.

ACKNOWLEDGEMENTS

We thank our students Mr. Patrizia Di Gennaro, Ada Festa, Simona Mariani and Flavio Rubiero for their collaboration in the experimental work.

REFERENCES

- 1 P. Falagiani, E. Cavallone, M. Nali, B. Rindone, S. Tollari and G. Crespi, *J. Chromatogr.*, 328 (1985) 425.
- 2 E. Bolzacchini, G. Di Gregorio, M. Nali, B. Rindone, S. Tollari, P. Falagiani, G. Riva and G. Crespi, *J. Chromatogr.*, 397 (1987) 299.
- 3 N. Rubio and A. Brieva, *J. Chromatogr.*, 407 (1987) 408.
- 4 S. Feo, R. Cochiara and D. Geraci, *Mol. Immunol.*, 21 (1984) 25.
- 5 S. A. Ford, B. A. Baldo, D. Geraci and D. Bass, *Int. Arch. Allergy Appl. Immunol.*, 79 (1986) 120.
- 6 D. Geraci, K. B. Billesbolle, R. Cocchiara, R. Lowenstein and H. Ipsen, *Int. Arch. Allergy Appl. Immunol.*, 78 (1985) 421.
- 7 A. L. Corbi and J. Carreira, *Int. Arch. Allergy Appl. Immunol.*, 74 (1984) 318.
- 8 A. L. Corbi and J. Carreira, *Int. Arch. Allergy Appl. Immunol.*, 76 (1985) 156.
- 9 E. Puttonen and H. J. Maasch, *J. Chromatogr.*, 242 (1982) 153.
- 10 S. Haavik, B. S. Paulsen and J. K. Wold, *J. Chromatogr.*, 321 (1985) 199.
- 11 E. Bolzacchini, G. Di Gregorio, M. Nali, B. Rindone, S. Tollari, P. Falagiani, G. Riva and G. Crespi, *Allergy*, 42 (1987) 743.
- 12 A. Bassoli, E. Bolzacchini, F. Chioccare, G. Di Gregorio, B. Rindone, S. Tollari, P. Falagiani and G. Riva, *J. Chromatogr.*, 446 (1988) 179.
- 13 M. Ceska, R. Erikson and J. M. Varga, *J. Allergy Clin. Immunol.*, 49 (1972) 1.
- 14 L. Yman, G. Ponterius and R. Brand, *Dev. Biol. Stand.*, 29 (1975) 151.
- 15 D. H. Calam and J. Davidson, *J. Chromatogr.*, 218 (1981) 581.