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Size-exclusion liquid chromatography and capillary electrophoresis of pollen allergens

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Abstract

Allergens from the pollen of *Phleum pratense*, *Dactylis glomerata*, *Arrhenatherum elatius*, *Secale cereale*, *Lolium perrene* and *Festuca* sp. were analysed by size-exclusion chromatography (SEC) and capillary electrophoresis (CE). SEC was used for the determination of the molecular masses of main allergens. A CE method, using either 150 mmol/l phosphoric acid (pH 1.8) or a micellar system consisting of 50 mmol/l sodium dodecyl sulphate–20 mmol/l borate (pH 9.35), was developed as a rapid and efficient alternative to SEC, especially for process control of allergenic preparations. The results obtained by the two methods confirmed similarities in the structures of the studied pollen allergens.

Keywords: Pollen allergens; Allergens

1. Introduction

Allergies belong among the most common diseases all over the world. In their treatment, allergen preparations obtained by extraction from natural materials are used. The raw extracts contain the allergens proper, which mostly are proteins or glycoproteins with molecular masses between 10 000 and 50 000, and other substances, such as peptides, glycosides and various low-molecular-mass compounds. Therefore, allergen extracts must be characterized and standardized [1].

The biological activity of allergens is determined by skin tests and immunochemical methods, whereas chromatographic techniques are employed for purification and isolation purposes. Size-exclusion chromatography (SEC) is one of the most popular methods and permits the separation of allergens and the determination of their molecular masses with reasonable reliability, provided that a suitable calibration procedure is employed [2,3]. Ion-exchange and reversed-phase HPLC are also used for these purposes, as are various electrophoretic methods.

Relatively little attention has so far been paid to capillary electrophoresis (CE), whose importance in the analysis of peptides and proteins is without doubt. CE has been used to analyse bee [4] and wasp [5] venoms and in the control of the production of allergen preparations [5].

This work was concerned with the use of SEC and CE in characterization of pollen allergens. These compounds cause some of the most widely

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occurring allergies in Europe. A great deal of attention has been paid to the allergens from *Phleum pratense* pollen [2,6–12] and it has been found that the principal allergen, Ph p I, is a glycoprotein occurring in various isoforms. It has further been found that the pollens of various grass species exhibit considerable immunochemical and chemical similarity [9,13–16].

2. Experimental

2.1. Chemicals

Samples of pollen extracts, *Dactylis glomerata*, *Arrhenatherum elatius*, *Secale cereale*, *Lolium perrene*, *Festuca* sp. and fractions of *Phleum pratense* isolated by gel chromatography were obtained from Sevac (Prague, Czech Republic).

Molecular mass standards ferritin (440 000), catalase (232 000), aldolase (158 000), human serum albumin (HSA) (68 459) ovalbumin (45 000), tripsinogen (24 000), myoglobin (17 400), ribonuclease A (13 700), aprotinin (6500) and vitamin B_{12} (1355), were obtained from Sigma (St. Louis, MO, USA). All other chemicals were of analytical-reagent grade from Lachema (Brno, Czech Republic) and were used without further purification.

2.2. Instrumentation and experimental conditions

HPLC measurements were performed using a

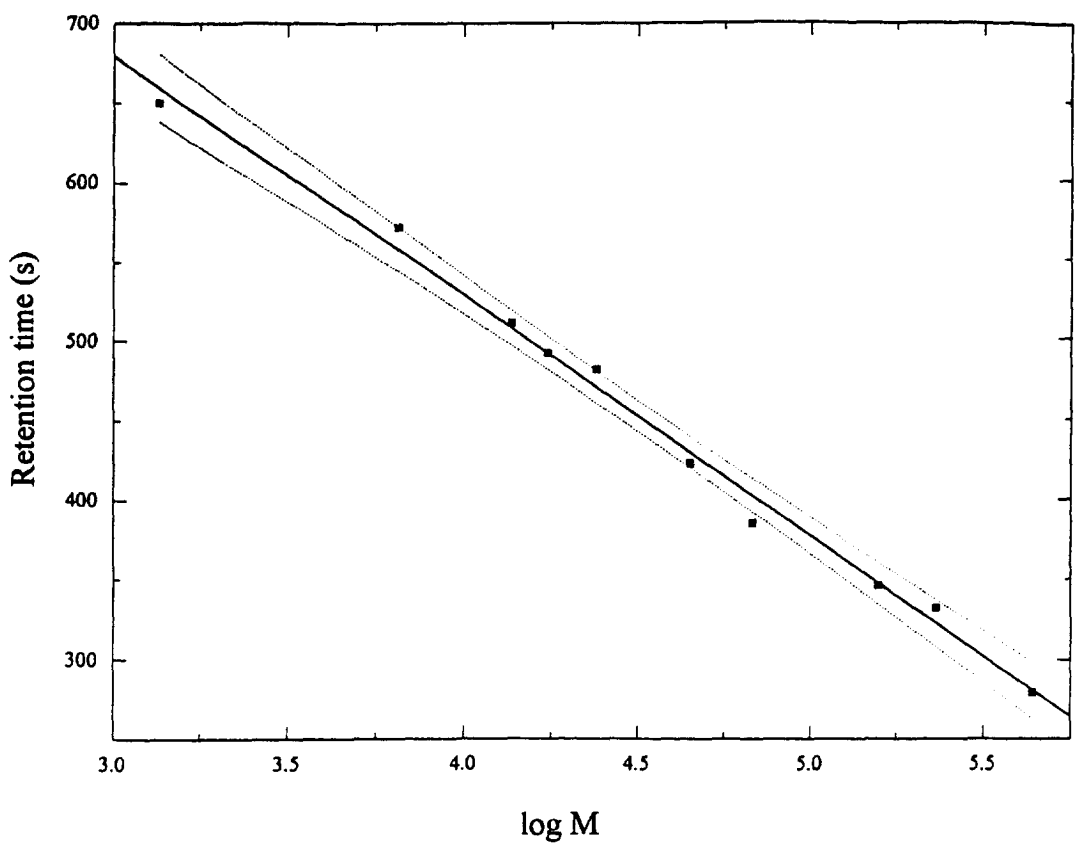

Fig. 1. Calibration of TSK 62000 SW column. For conditions, see Experimental. The dotted lines indicate the confidence interval at a significance level $\alpha = 0.05$.

Crystal 200 liquid chromatograph with Crystal 250 diode-array detector (ATI Unicam, Cambridge, UK) and a Model 7125 injector (Rheodyne, Cotati, CA, USA). The data were collected and processed using a Dell data station with a Unicam 4880 chromatographic data handling system.

For SEC, a steel column (300 × 7.5 mm I.D.) containing 10-μm TSK G2000 SW Ultropac packing (LKB, Bromma, Sweden), was used at a flow-rate of 1.0 ml/min. The mobile phase was 0.2 mol/l phosphate buffer–1.0 mol/l KCl (pH 7.4).

CE was carried out on a Crystal Model 310 CE instrument with a variable-wavelength UV photometric detector (ATI Unicam). The total length of the fused-silica capillary was 75 cm, the length to the detector was 61 cm and the I.D. was either 50 or 75 μm. A constant potential of 20 kV was applied. UV detection at 190 nm was used. Samples were injected pneumatically, with an overpressure from 30 to 100 mbar for 0.05–0.1 s. The electrolyte systems were (a) 150 mmol/l phosphoric acid (pH 1.8) and (b) 20 mmol/l borate buffer–50 mmol/l sodium dodecyl sulphate (SDS) (pH 9.35).

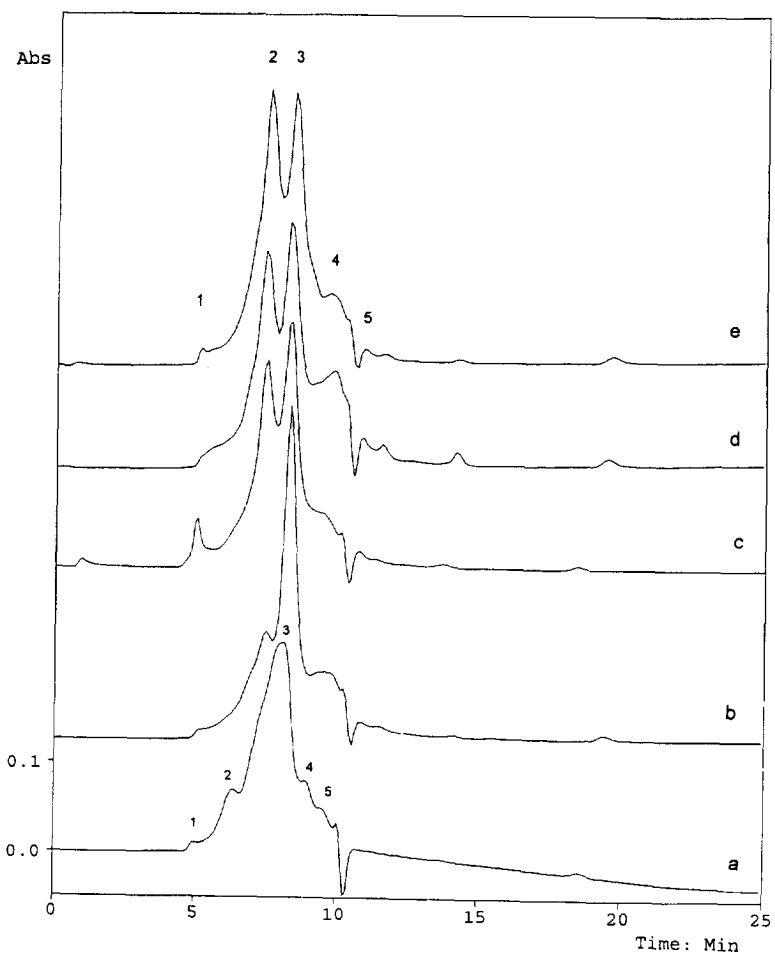

Fig. 2. SEC of pollen extracts: (a) *Secale cereale*; (b) *Phleum pratense*; (c) *Dactylis glomerata*; (d) *Festuca* sp.; (e) *Arrhenatherum elatius*. For conditions, see Experimental; $\lambda = 213$ nm.

The samples were dissolved in the running buffer for SEC and in deionized water for CE.

3. Results and discussion

3.1. Size-exclusion chromatography

SEC was primarily used to determine the molecular masses of the components of the pollen allergen preparations, which can be considered as their principal characteristic. The TSK G2000 SW column used is hydrophilic and permits separations of substances of molecular masses not exceeding 400 000. The column was calibrated with a standard protein mixture (Fig. 1). The parameters of the equation

$$t_R = a - b \log M \quad (1)$$

where t_R = retention time (s) and M = molecular mass, were determined, obtaining the values a = 1132.2, $s_a = 20.7$ and $b = 150.9$, $s_b = 4.49$, with correlation coefficient $r = -0.9965$. The relative standard deviation of the retention times was between 0.4 and 0.8%; the confidence interval at a significance level of $\alpha = 0.05$ (Fig. 1) permits the determination of the molecular masses with an error of ±1000. An example of the separation of various dialysed and lyophilized pollens is shown in Fig. 2 and the molecular masses obtained for the main components are listed in Table 1.

The comparison of the chromatograms of various pollen allergens in Fig. 2 demonstrates significant similarities in their composition. The main components are substances with molecular

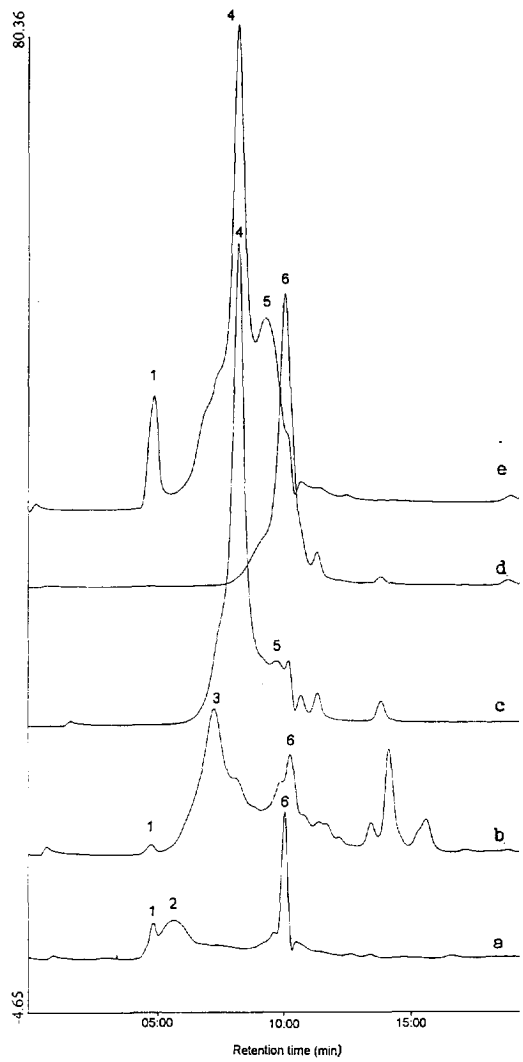

Fig. 3. SEC of *Phleum pratense* pollen extracts: (a) fraction I; (b) fraction II; (c) fraction III; (d) fraction IV; (e) raw dialysed pollen extract. For conditions, see Experimental.

Table 1
Molecular masses of pollen extract components

Component	*Phleum pratense*	*Dactylis glomerata*	*Festuca* sp.	*Secale cereale*	*Arrhenatherum elatius*
1	–	330 000	–	320 000	310 000
2	34 000	35 000	35 000	24 000	33 000
3	17 000	17 000	17 000	16 000	15 000
4	5000	5300	3700	9000	5000
5	2500	2500	1800	4000	2000

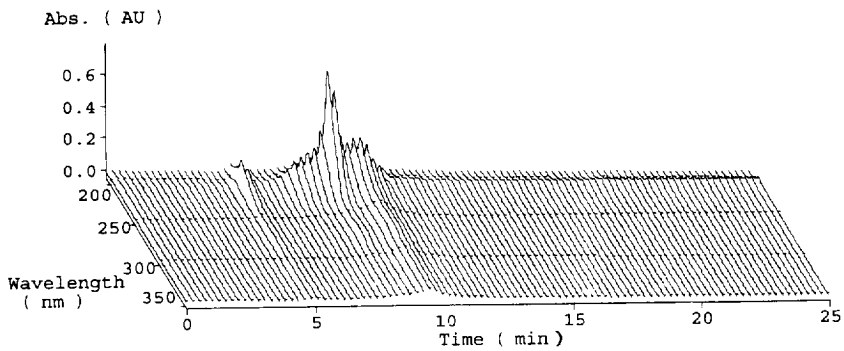

Fig. 4. SEC of *Phleum pratense* pollen with diode-array detection. For conditions, see Experimental.

masses around 34 000 (No. 2) and 17 000 (No. 3) and their contents in the pollens of *Dactylis glomerata*, *Festuca* sp. and *Arrhenatherum elatius* are roughly the same. The *Phleum pratense* pollen contains significantly more of component No. 3 than No. 2. All the pollens further contain substances with lower molecular masses (around 5000). The rye (*Secale cereale*) pollen differs from the other pollens. Its components are insufficiently separated and the main component has a lower molecular mass (24 000) than those from the other pollens.

The greatest attention was paid to the *Phleum pratence* pollen, in view of its importance in our geographical area. The raw extract was dialysed and separated by gel chromatography into four fractions whose SEC analysis is depicted in Fig. 3. Skin tests, carried out by Dr. Brož at Šumperk Hospital, indicated the highest activity of fraction III containing the component with a molecular mass of 17 000 (Table 1). Diode-array detection permitted the optimum detection wavelength to be obtained and evaluation of the peak purity. It was found that the main allergen is a glycoprotein exhibiting maximum absorbance at 213 nm (Fig. 4); the absorbance was very low in the region of protein absorption (270–280 nm).

3.2. Capillary electrophoresis

A number of separation systems were tested and the best results were obtained with the following two:

(a) In 150 mmol/l phosphoric acid (pH 1.8) the electroosmotic flow is very low and the capillary walls are positively charged (the silanol

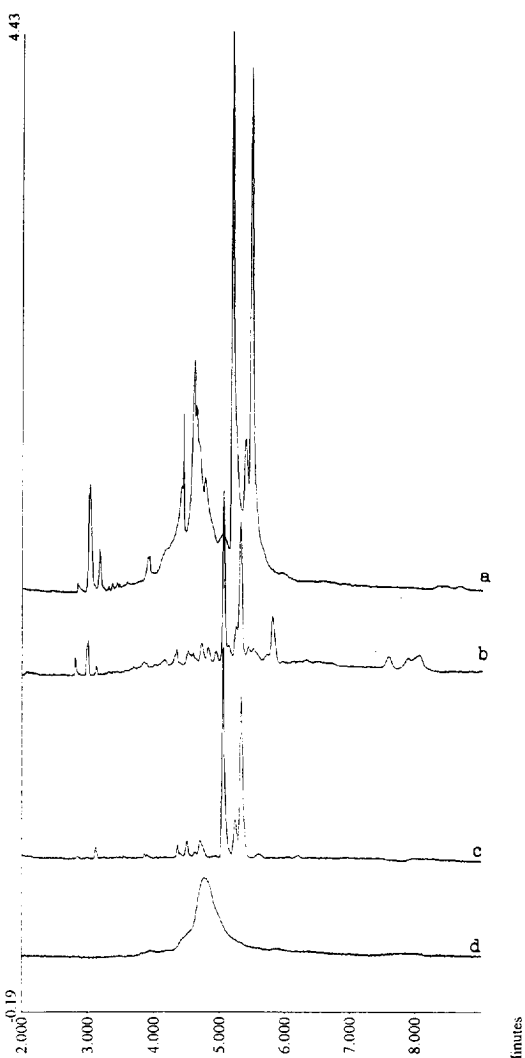

Fig. 5. CE separation of *Phleum pratense* pollen extract [150 mmol/l phosphoric acid (pH 1.8)]: (a) raw extract; (b) ultrafiltrate; (c) fraction IV; (d) fraction III.

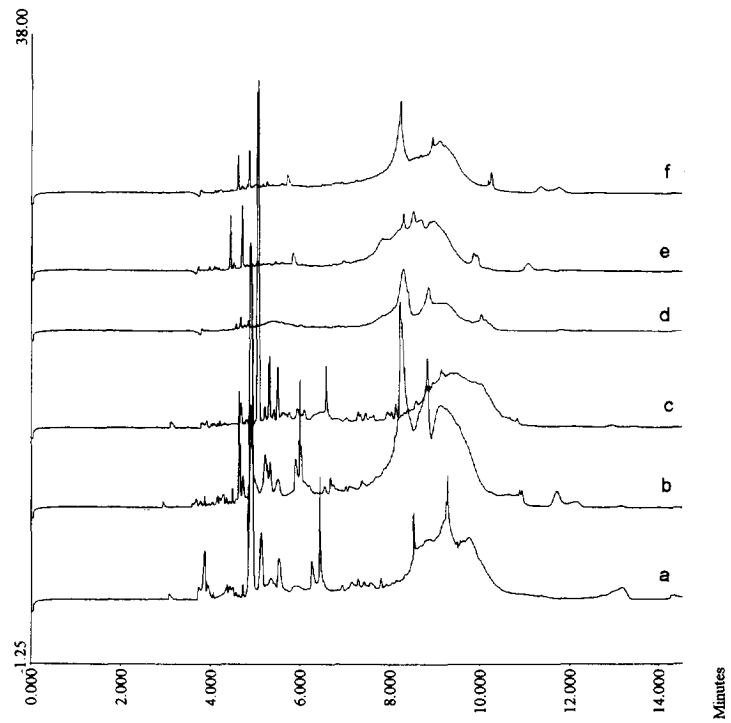

Fig. 6. CE separation of various pollen extracts [50 mmol/l SDS–20 mmol/l borate (pH 9.35)]: (a) *Lolium perrene*; (b) *Dactylis glomerata*; (c) *Phleum pratense*; (d) *Arrhenaterum elatius*; (e) *Secale cereale*; (f) *Festuca* sp.

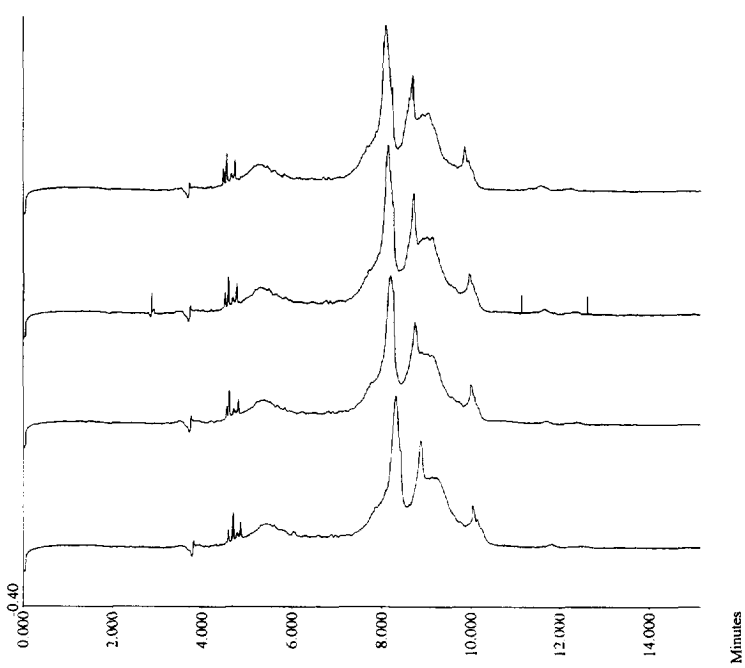

Fig. 7. Quality control of different batches of *Arrhenatherum elatius* allergen products in vitro by CE [50 mmol/l SDS–20 mmol/l borate (pH 9.35)].

groups are protonated), so that adsorption of proteins and glycoproteins on the walls is suppressed; an example of separation is given in Fig. 5.

(b) A micellar system, containing 50 mmol/l SDS and 20 mmol/l borate (pH 9.35), permits the separation of all the studied pollen allergens, as shown in Fig. 6. The broad peak corresponds to the main allergen, a glycoprotein, which is present in several isoforms. Fig. 6 demonstrates the similarity among the studied pollen allergens.

UV photometric detection exhibited the highest sensitivity at 190 nm in both of the above separation systems.

The CE method is suitable for the control of the quality of allergen preparations, as demonstrated in Fig. 7 for the analyses of four products containing the *Arrhenantherum elatius* pollen allergens in vitro.

4. Conclusions

SEC is a reliable and reproducible method for separations of components of allergenic materials and for the determination of the molecular masses of the components. CE is a very rapid, highly efficient separation method with a very low consumption of chemicals and the samples, which are especially well suited for production control of allergen preparations.

Acknowledgement

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