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Analysis of liquid extracts from tree and grass pollens by capillary electromigration methods

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Abstract

Capillary electromigration methods, zone electrophoresis (CZE), micellar electrokinetic chromatography (CMEKC) and isotachophoresis (CITP), have been used for analysis of water and water–buffer extracts from tree—common birch (*Betula verrucosa*) and grass—orchardgrass (*Dactylis glomerata*) pollen samples. Water extracts were analyzed by CZE using acetic acid as background electrolyte (BGE), by CMEKC in tris-phosphate BGE with anionic detergent sodium dodecyl sulfate (SDS) micellar pseudophase (TP-SDS) and by CITP in cationic mode with leading/terminating cations K^+/BALA$^+$ (β-alanine (BALA)) and in anionic mode with leading/terminating anions Cl^-/MES$^-$ (2-(*N*-morpholino)ethanesulphonic acid (MES)). Moreover, acetic acid extracts were analyzed by CZE using acetic acid as BGE, and alkaline water–SDS-buffer extracts were analyzed by CMEKC using TP-SDS as BGE. Extracted amounts of pollen allergens and other UV-absorbing compounds and the number of resolved components were evaluated from CZE, CMEKC and CITP analyses of the liquid extracts. Larger amounts of UV-absorbing material were found in the water–buffer pollen extracts than in the water extracts. More UV-absorbing material was found in all extracts from *D. glomerata* pollen than in relevant extracts from *B. verrucosa* pollen. It was found by CITP that the extracted amounts of anionic components and their number were much higher than those of cationic components. Concentrations of some inorganic ions (e.g. Cl^-, K^+, Na^+, Ca^{2+}) in pollen samples were also determined by CITP.

Keywords: *Betula verrucosa*; *Dactylis glomerata*; Pollen allergens

1. Introduction

In recent decades, an increased prevalence of allergic and asthmatic individuals has been observed in human populations in the industrially developed countries. Pollen allergens, particularly those from grass, weed or tree pollens, belong to the main out-door air allergens and they are considered to be responsible for a great part of allergic diseases of respiratory tract.

There are some indications that the effect of pollen allergens is amplified by some types of the air pollution, namely its fine and ultrafine particulate load [1]. Pollutants resulting from burning of fossil fuels and automobile exhaust gas, especially diesel exhaust particles (DEPs), are of special interest because of their widespread occurrence [2]. Air pollutants may not only increase the frequency and intensity of symptoms in already allergic patients but may promote airway sensitization to airborne allergens in predisposed subjects. Pollutants can modify the morphology of pollen grains by attaching to their surface [3]. It was shown that there is a direct in situ interaction between grass (*Dactylis glomerata*) pollen grains surfaces and airborne particulate matter (APM) [4]. Aqueous extracts of APM induce the release of pollen allergens with altered antigenicity. This effect is prominent in industrialized regions with high emission of organic pollutants and near roads with heavy traffic. Thus, pollens may be used as sensitive biological indicators of atmospheric pollution [5]. It has also been shown that grass pollen allergen molecules bind specifically onto DEPs [6]. In mice, DEPs have been shown to enhance the IgE response to birch pollen [7], and traffic particulate matter, collected in Prague tunnel, has been demonstrated to affect both the inflammatory and immunological components of experimental birch pollen allergy [8].

In pollen traps, only intact pollen grains could be recognized by counting them under a microscope. The data are not available early enough for prophylactic measures. Furthermore, ordinary pollen counts do not reflect the

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concentration of damaged pollen grains, i.e. grain fragments still bearing allergenic molecules. It is thus relevant to measure the concentration of airborne pollen antigens rather than that of pollen grains for prevention of allergic reactions in man [9].

From these facts, it follows that the simultaneous detection and quantification of pollen allergens and organic pollutants in the air are necessary for a complex evaluation of the influence of environmental factors on the allergy and asthma, and for the early warning of concerned people on the appearance of the elevated concentrations of both allergens and pollutants in the air. Consequently, the final aim of our work is to contribute to build an automated air trap, which will collect the air dust and at regular intervals will extract pollen allergens and organic pollutants and quantify them. The partial aim is to use the capillary electromigration methods for the analysis of these extracts, for evaluation of the efficiency of the extraction of pollen allergens by different extracting procedures and agents.

Different modes of capillary electrophoresis have developed into highly-efficient and highly-sensitive analytical methods with a broad application potential for analysis of various classes of compounds of environmental relevance, including pollen allergens [10,11] and organic pollutants [12]. Capillary isotachophoresis (CITP) [13] plays a specific role in environmental analysis because of possessing the concentrating effect, i.e. concentration of the sample components in ITP steady state is adapted to that of the leading ion. In this way the originally diluted analyte ions are concentrated into sharply separated zones with stepwise concentration changes. For that reason CITP is often applied as a concentration pretreatment for capillary zone electrophoresis (CZE) in environmental analysis. There are many application of CITP in organic and inorganic environmental trace analysis, for a review see [14]. CITP was also applied to the identification and characterization (profiling) of various allergenic extracts including pollens [15,16].

Recently, we have demonstrated that capillary electromigration methods, namely CZE, capillary micellar electrokinetic chromatography (CMEKC) and CITP, can be used for evaluation of the efficiency of extraction of pollen allergens and organic pollutants from the airborne dust samples [17,18].

The aim of this paper was to test the applicability of capillary electromigration methods, CZE, CMEKC and CITP, for analysis of the liquid extracts of pollen samples, to evaluate the efficiency of different extraction procedures (extraction agents) by the determination of the amount of extracted allergens from tree and grass pollen samples, common birch (*Betula verrucosa*) and orchardgrass (*D. glomerata*), respectively, and to find out the differences in the qualitative and/or quantitative composition of the extracts of these grass and tree pollens. In addition, from the CITP analyses of water extracts, the concentrations of some inorganic anions and cations in the pollen samples should be determined.

2. Experimental

2.1. Chemicals

All chemicals used were of analytical reagent grade. Phosphoric, hydrochloric and acetic acids, potassium hydroxide and acetone were from Lachema (Brno, Czech Republic). Tris(hydroxymethyl)aminomethane (Tris), 2-(*N*-morpholino)ethanesulphonic acid (MES) and sodium dodecyl sulfate (SDS) were from Serva (Heidelberg, FRG). D,L-Histidine was obtained from Sigma (St. Louis, MO, USA), and β-alanine (BALA) from Calbiochem (Luzerne, Switzerland). Buffer solutions were prepared from the deionized and redistilled water and filtered through 0.45 μm membrane filter (Millipore, Bedford, USA) prior to the use in capillary electromigration methods.

2.2. Pollen extraction procedures

Pollen samples from tree (*B. verrucosa*) and grass (*D. glomerata*) were obtained from Allergon (Angelholm, Sweden).

Three different extraction procedures using the following extracting agents were tested:

(i) water;
(ii) acid water solution (0.5 M acetic acid, pH 2.5); and
(iii) alkaline water–buffer solution with anionic detergent (20 mM Tris, 5 mM H_3PO_4, 50 mM sodium dodecyl sulfate, pH 8.7).

In all three procedures, 50 mg of the pollen sample was suspended into 0.45 ml of extracting agent, extraction was performed during gentle turning at ambient temperature 23 °C for 1 h followed by centrifugation at 10 000 rpm (r = 66 mm) for 5 min. The yield was 0.25 ml of liquid extract.

2.3. Capillary zone electrophoresis and micellar electrokinetic chromatography

CZE and CMEKC experiments were performed in home-made apparatus equipped with UV photometric detector monitoring absorbance at 206 nm. Data acquisition and handling were performed using the CSW 32 chromatography station (DataApex, Prague, Czech Republic). Fused silica capillary (i.d. 50 μm, o.d. 200 μm, total length 315 mm, effective length 200 mm) was supplied by the Institute of Glass and Ceramics Materials, Czech Academy of Sciences (Prague, Czech Republic). Separations were performed at ambient temperature 22–24 °C. Sample solution was applied hydrodynamically (pressure 500 Pa for 5–10 s). The applied voltage (constant) was 10.0 kV and current 8 μA in CZE and 22 μA in CMEKC. The background electrolytes (BGEs) were 0.5 M acetic acid, pH 2.5 (HAc) for CZE, and 20 mM Tris, 5 mM H_3PO_4, 50 mM SDS, pH 8.7 (TP-SDS) for CMEKC.

Table 1
Composition of CITP electrolyte systems

CITP mode	Leading electrolyte (LE)			Terminating electrolyte (TE)		
	Leading ion	Counterion	pH	Terminating ion	Counterion	pH
Anionic	10 mM Cl^-	20 mM His^+	5.8	10 mM MES^-	H^+	3.7
Cationic	10 mM K^+	25 mM Ac^-	4.4	10 mM $BALA^+$	Ac^-	4.4

2.4. Capillary isotachophoresis

Capillary isotachophoretic experiments were performed in the electrophoretic analyzer EA 101 (Villa Labeco, Spišská Nová Ves, Slovakia) equipped with column coupling system consisting of two fluorinated ethylene–propylene copolymer (FEP) capillaries. The first, preseparation capillary (160 mm × 0.8 mm i.d.) is connected with the analytical capillary (180 mm × 0.3 mm i.d.) via the bifurcation block, which enables determination of the sample macrocomponents in the preseparation capillary and determination of microcomponents in the analytical capillary. Contactless conductivity detectors are placed on both columns 40 mm from the outlet ends, and UV-absorption detector (set to the wavelength 254 nm) is situated 30 mm from the outlet end of the analytical column. Sample volumes 2–5 μl were applied by microsyringe through the septum above the injection valve. The driving currents were 250 and 50 μA in the preseparation and analytical columns, respectively. Separations were performed at ambient temperature 22–24 °C. The composition of electrolyte systems used is given in Table 1.

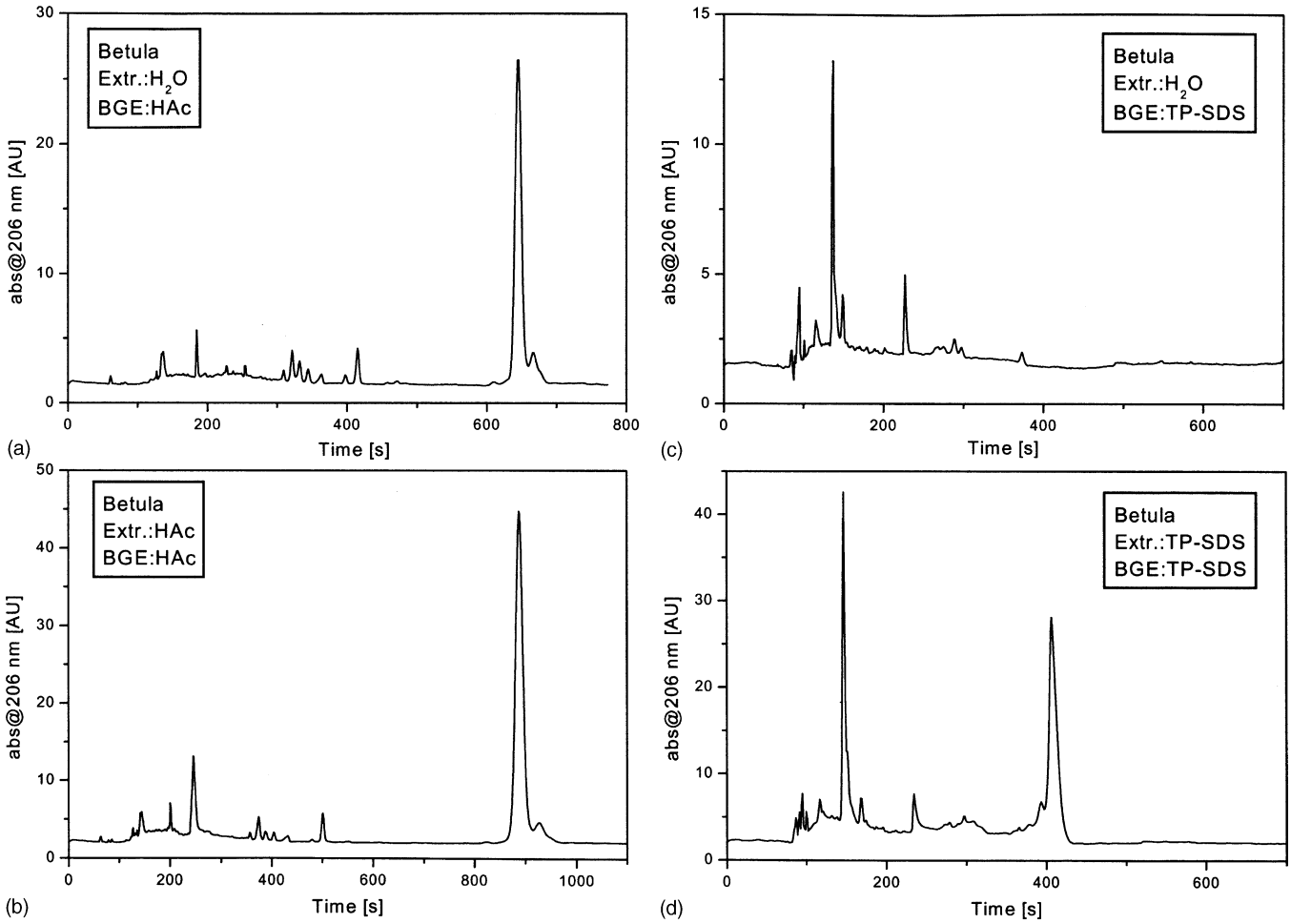

Fig. 1. CZE and CMEKC analyses of the liquid extracts from the pollen samples of *Betula verrucosa*: (a) water extract and (b) HAc extract—both analyzed by CZE in HAc BGE; (c) water extract and (d) TP-SDS extract—both analyzed by CMEKC in TP-SDS BGE. HAc: 0.5 M acetic acid, pH 2.5; TP-SDS: 20 mM Tris, 5 mM H_3PO_4, 50 mM SDS, pH 8.7; abs@206: absorbance at 206 nm in arbitrary units (other experimental conditions are given in the text, Section 2.3).

Table 2
Extracted amount (in relative arbitrary units, AU) of pollen allergens and other UV-absorbing components and their number in the extracts from *Betula verrucosa* and *Dactylis glomerata* pollen samples extracted by different extraction agents and analyzed by CZE and CMEKC methods in HAc and TP-SDS background electrolytes (BGEs)

Pollen sample	Extracting agent	BGE	Extracted amount (AU)	Number of found components
Betula verrucosa	Water	HAc	1394	51
		TP-SDS	1011	28
	HAc	HAc	3420	58
	TP-SDS	TP-SDS	3371	33
Dactylis glomerata	Water	HAc	1751	47
		TP-SDS	2280	37
	HAc	HAc	4146	52
	TP-SDS	TP-SDS	4497	38

HAc: 0.5 M acetic acid, pH 2.5; TP-SDS: 20 mM Tris, 5 mM H_3PO_4, 50 mM SDS, pH 8.7.

3. Results and discussion

Capillary electromigration methods, CZE and CMEKC, have been used for analysis of water and water–buffer extracts of the pollen samples from common birch (*B. verrucosa*) and orchardgrass (*D. glomerata*). The water extracts and the acid water–buffer solution extracts were analyzed by CZE in the acid background electrolyte, 0.5 M acetic acid, pH 2.5 (further indicated as HAc; see Figs. 1a,b (*Betula*) and 2a,b (*Dactylis*)). Water extracts and alkaline water–SDS-buffer extracts were analyzed by CMEKC using 20 mM Tris, 5 mM H_3PO_4, 50 mM SDS, pH 8.7 (further

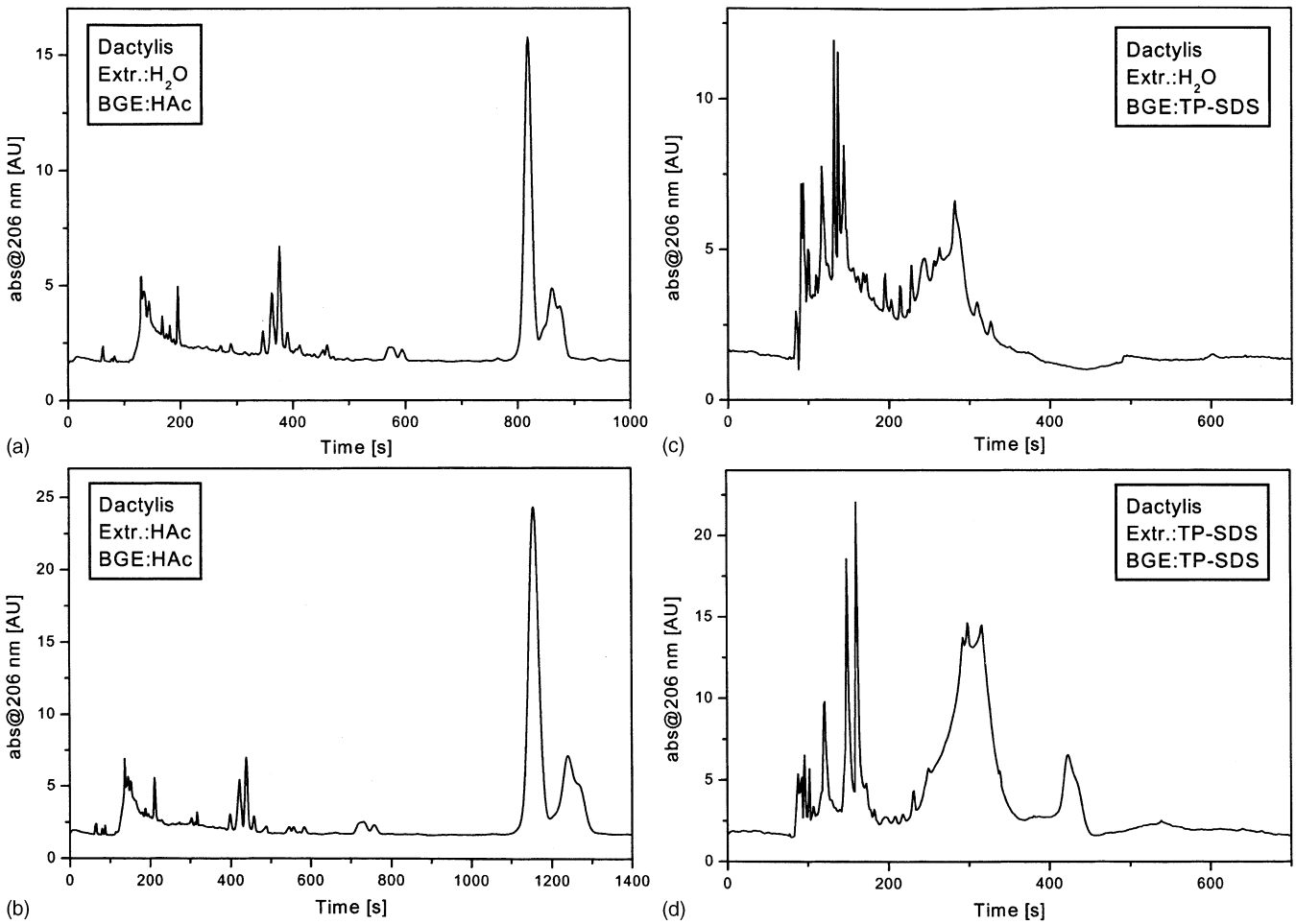

Fig. 2. CZE and CMEKC analyses of the liquid extracts from the pollen samples of *Dactylis glomerata*: (a) water extract and (b) HAc extract—both analyzed by CZE in HAc BGE; (c) water extract and (d) TP-SDS extract—both analyzed by CMEKC in TP-SDS BGE (other conditions as in Fig. 1).

indicated as TP-SDS) as BGE (50 mM SDS forms the micellar pseudophase of this BGE; see Figs. 1c,d (*Betula*) and 2c,d (*Dactylis*)).

The amount of extractable material has been evaluated by integration of the electrophoregrams and chromatograms of CZE and CMEKC analyses of the pollen extracts. The amount of extracted material is directly proportional to the total area of UV-positive peaks (measured at 206 nm). The number of found components in different extracts has been also approximately estimated as the number of resolved peaks from the corresponding records of CZE and MEKC analyses. The results are presented in Table 2.

Comparing the extracted amounts from the same pollen sample, obtained by the different extraction procedures (agents), the efficiency of the extraction was evaluated. More material was extracted in the water–buffer extracts than in the water extracts. The amount of UV-absorbing material extracted by TP-SDS correlates with finding [19] that by extraction with alkaline medium (pH 8.5), the protein content of birch pollen extracts is much higher in comparison with that in water extracts.

More components were resolved in HAc extracts from both pollens than in water extracts. More UV-absorbing material was found in all extracts from *D. glomerata* pollen than in relevant extracts from *B. verrucosa* pollen. Some similarities have been found in the analyses of extracts from the pollen samples from both species (see Figs. 1 and 2), e.g. the highest peak of the low-mobility component and similar profiles of high- and medium-mobility components in HAc BGE.

Moreover, the water extracts were also analyzed by CITP both in anionic and cationic modes using the electrolyte systems given in Table 1. The example of conductivity detector records (integral plot and 1st derivative; cationic and anionic modes) of CITP analyses of water extracts from *D. glomerata* pollen sample are shown in Fig. 3. In addition, to some "classical", stepwise and well-resolved ITP zones of macrocomponents present in the sample, apparently several zones of minor components are not sufficiently resolved by the contactless conductivity detector due to their short lengths, small differences in conductivity and a relatively lower resolution capability of the contactless conductivity detector than that of UV-absorption detector. For that reason and also taking into account the fact that components of pollen allergens are UV-absorbing compounds (proteins and glycoproteins), the amount of the pollen extractable material has been evaluated from the UV-detector records at 254 nm of CITP analyses of the pollen extracts. UV-absorption isotachophoregrams of anionic and cationic CITP analyses of the water extracts of the pollen *B. verrucosa* and *D. glomerata* are presented in Fig. 4. Obviously, the amount of extracted material is proportional to the total area of UV-positive peaks related to migration velocity of ITP zones. The number of resolvable components has been also estimated from the UV-absorption records of CITP analyses. The results are presented in Table 3.

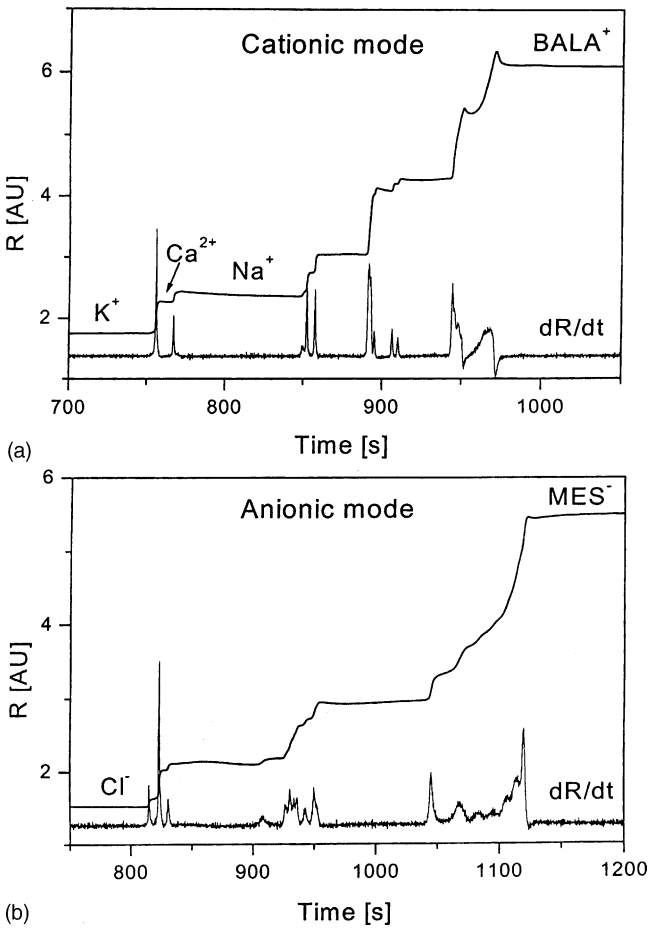

Fig. 3. Conductivity detector records (integral plot and 1st derivative) of CITP analyses of water extracts from the pollen sample of *Betula verrucosa*: (a) cationic mode; (b) anionic mode; *R*: resistance in arbitrary units; d*R*/d*t*: 1st derivative of conductivity detector signal (sample volume 3 μl; other experimental conditions are given in the text, Section 2.4.).

Table 3
Extracted amount (in relative arbitrary units, AU) of pollen allergens and other UV-absorbing components (λ = 254 nm) and number of components found in the water extracts of pollens from *Betula verrucosa* and *Dactylis glomerata* determined by CITP

Pollen sample	Anionic mode		Cationic mode	
	Extracted amount (AU)	Number of components found	Extracted amount (AU)	Number of components found
Betula verrucosa	133.0	23	27.9	15
Dactylis glomerata	300.0	24	53.4	19

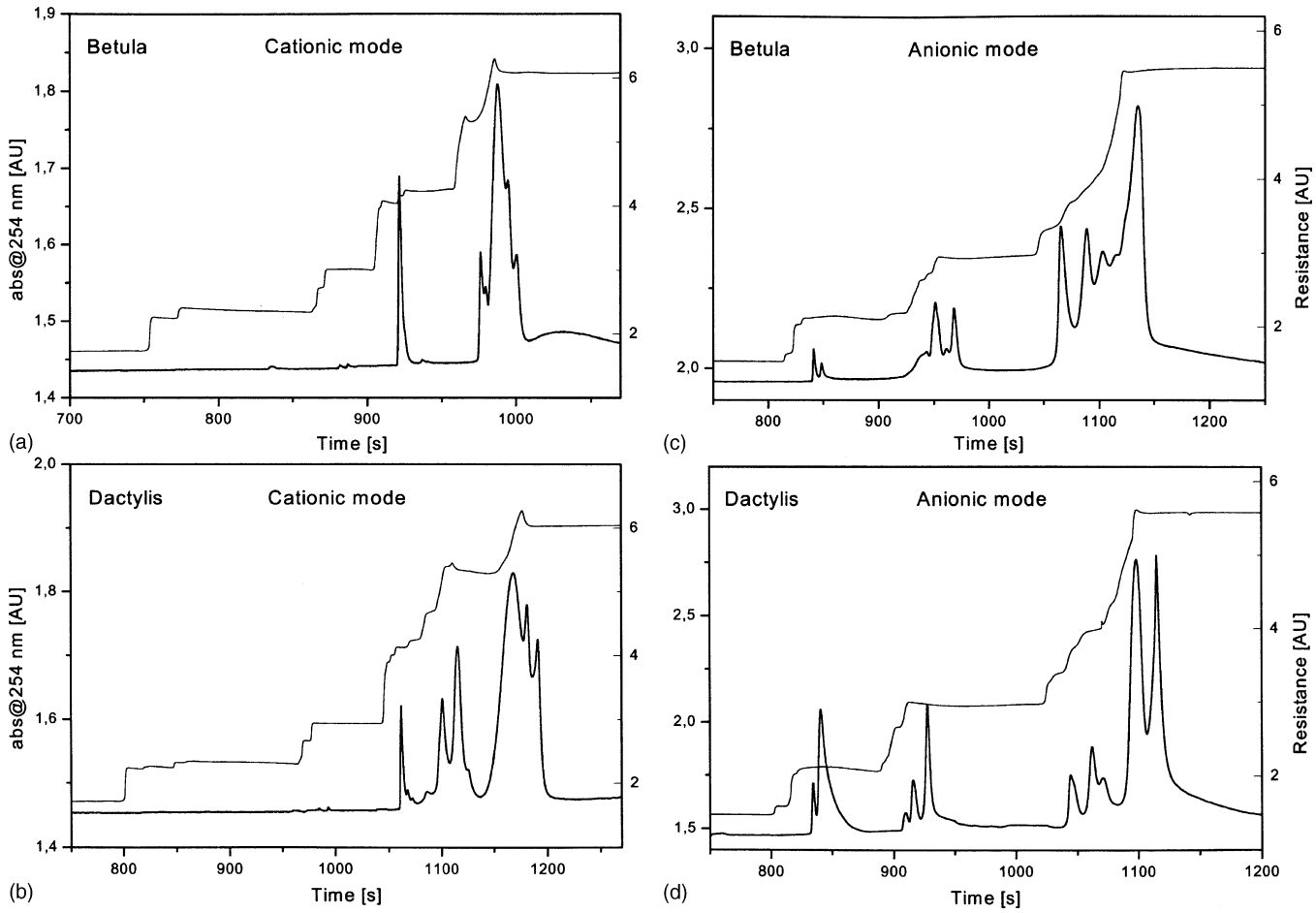

Fig. 4. CITP analyses of water extracts from pollen samples of *Betula verrucosa* and *Dactylis glomerata*. (a and b) Cationic mode; (c and d) anionic mode; upper curves: conductivity detector records; lower curves: UV-detector records; abs@254: absorbance at 254 nm in arbitrary units (sample volumes 3 μl; other conditions as in Fig. 3).

The substance amounts of ions in the pollen samples, which are identical with the leading ions (chlorides in anionic mode, potassium ions in cationic mode), n_L, were calculated from the leading ion concentration, c_L, capillary cross-section, S, and from the prolongation of the leading electrolyte (LE) zone lengths in the experiments with the samples in comparison with the LE zone length without sample, determined from the prolonged migration time of the boundary between LE and the first sample zone, Δt, and from migration velocity of LE, v:

$$n_L = c_L S v \, \Delta t$$

The substance amounts of the ions were converted to mass amounts in the injected sample volumes and related to the mass unit of the dried pollen sample before water extraction. The substance amounts of sodium and calcium ions in water extracts were determined from the corresponding calibration curves, the equations of which are presented in Table 4. The substance amounts of these ions in applied sample volumes were also converted to mass units and related to mass unit of the dried pollen. The concentrations (expressed in ppm units) of some inorganic ions, K^+, Na^+, Ca^{2+} and Cl^-, in the pollen samples *B. verrucosa* and *D. glomerata* are presented in Table 5.

Determination of the amount of extracted material from both pollen samples, obtained by the same extraction procedure, allowed to estimate and compare the content of UV-absorbing components (at 254 nm detection wavelength) in the pollen samples of *B. verrucosa* and *D. glomerata*, respectively. Larger amounts of extracted material and more resolved components were found in the water extracts of both pollen samples analyzed in anionic mode in comparison with those analyzed in cationic mode. Both

Table 4
Relative step heights (RSHs) and calibration curves equations of the CITP determination of calcium and sodium ions in cationic electrolyte system given in Table 1

Ion	RSH	Calibration curve equation	R^2_{xy}
Calcium	0.104 ± 0.009	$y = 5.04x - 0.50$	0.989
Sodium	0.128 ± 0.007	$y = 3.20x - 2.82$	0.998

y: zone length (s); x: amount of substance (nmol); R_{xy}: correlation coefficient.

Table 5
Determination of some inorganic ions in the pollen samples by CITP

Pollen sample	Concentration of selected ions in the pollen samples (ppm)[a]			
	K^+	Cl^-	Na^+	Ca^{2+}
Betula verrucosa	10029	469	987	238
Dactylis glomerata	19179	3449	1842	541

[a] Averaged values from two ITP analyses, the values of which differed less than 2–3%.

in anionic and cationic modes, larger amounts of extracted UV-absorbing material were found in *D. glomerata* pollen sample than in *B. verrucosa* one.

Some similarities have been found in the analyses of extracts from *Betula* and *Dactylis* samples, e.g. three "blocks" of UV-absorbing zones belonging to components with high, middle and low mobilities in anionic mode; see Fig. 4c and d. On the contrary, in cationic mode high- and low-mobility UV-absorbing zones are also present in both pollens, but the medium mobility block is present only in *D. glomerata* pollen; see Fig. 4a and b. In anionic mode, UV-absorbing components are distributed in the whole mobility range between leading and terminating electrolytes whereas in cationic mode the UV-absorbing components are present only at medium and lower mobility range between LE and TE. No special correlation has been found between the inorganic ions contents in pollen extracts.

4. Conclusions

Capillary electromigration methods, CZE, CMEKC and CITP, have been shown to be highly-efficient and highly-sensitive methods suitable for analysis of water and water–buffer extracts from tree and grass pollens, particularly for the estimation of the content of UV-absorbing components in the pollen samples and for the evaluation of the efficiency of the extraction procedures applied to extraction of pollen allergens. Larger amounts of extracted allergens and other UV-absorbing components were found in *D. glomerata* pollen sample extracts than in *B. verrucosa* ones.

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