ELSEVIER

Journal of Chromatography B, 699 (1997) 403–418

JOURNAL OF CHROMATOGRAPHY B

Review

High-performance separations in isolation and characterization of allergens

Věra Pacáková[a,*], Karel Štulík[a], Marie Tichá[b]

[a]*Department of Analytical Chemistry, Charles University, Albertov 2030, 128 40 Praha 2, Czech Republic*
[b]*Department of Biochemistry, Charles University, Albertov 2030, 128 40 Praha 2, Czech Republic*

Abstract

The present state of the use of separation techniques in the identification and characterization of allergens and in the monitoring of the quality of allergenic preparations is critically surveyed. After a brief summary of the range of problems encountered in obtaining and in the application of allergenic preparations and of the principal physico-chemical properties of allergens, chromatographic and electromigration methods of separation of components of these systems and their combinations with immunochemical procedures are discussed, with selected examples of application to real materials. Emphasis is placed on evaluation of the most important analytical parameters, such as reliability of the results, separation efficiency and resolution, and on the most recent results in the field. © 1997 Elsevier Science B.V.

Keywords: Reviews; Allergens

Contents

*Corresponding author.

PII S0378-4347(97)00223-5

1. Introduction

Allergies are among the most common diseases all over the world and their occurrence is constantly growing, causing increased concern and necessitating intense research into their origin, mechanism and approaches to their diagnosis, treatment and prevention. Allergology is a part of immunology and thus deals with very complex biological interactions, with many gaps in our knowledge.

The most important allergies (type I) are caused by antigens that lead, in sensitive individuals, to an increased production of antibodies from the immunoglobulin E (IgE) class. These antigens are called allergens and are contained in various natural sources, primarily in the pollen of some grasses and trees, the faeces of mites, animal epithelia, fungi, insect venoms, bacteria, as well as in many man-made products, e.g. foodstuffs or drugs. Allergens enter the organism through the mucous membranes of the respiratory and gastrointestinal tracts or through the skin and some of them, for example, the bee venom, can directly endanger human life [1]. The study of allergies and consequent diagnostic and therapeutic procedures must thus be based on close cooperation of physicians with biologists and chemists and must involve identification and physicochemical characterization of individual allergens, their preparation in a sufficiently pure form and a medical study of their effects on suitably selected sets of patients [2–6].

Crude allergen preparations are most often obtained by extraction of natural materials with water or aqueous buffers. As all the water-soluble substances are extracted, complex and ill-defined mixtures result containing proteins, glycoproteins, glycosides, peptides and various compounds of low molecular masses, such as amino acids, saccharides, biogenic amines and some inorganic substances.

The allergens proper are mostly proteins or glycoproteins with molecular masses in a range of ca. 5000 to 50 000 rel. mol. mass units, but some molecules outside this range have also been identified as allergenically active. The lower molecular mass limit is dictated by the requirement of a sufficient molecular complexity in order to elicit an immune reaction and the upper limit is given by the size of the molecule that still permits its penetration through a mucous membrane [2]. The allergens are readily soluble in physiological buffers as are all the globular proteins and their isoelectric points are between 3 and 11. Surface carbohydrates on proteins are responsible for many biological functions that are connected with molecular recognition and thus the determination of glycoprotein heterogeneity is very important. The contents of saccharide moieties vary from zero to pure polysaccharides; the latter occur, e.g., in the fungi *C. herbarum* and *A. fumigatus* [7]. Any chemical change in the allergen molecule results in a change in its allergenic activity; in this respect, the amino acid sequence of the proteins is of importance. The thermal stability of allergens, which varies for various compounds, must also be considered.

The optimal solution to the problem of the study and use of allergenic preparations is to obtain a pure specific allergen, to establish its chemical structure, to measure its concentration and to follow its biological activity. The pure allergen content can then be directly correlated with the allergenic activity and can replace the conventional biological units that are commonly used with more complex allergenic preparations [8]. However, this task is very difficult. First of all, crude allergen extracts contain not only major allergens, but also minor ones; moreover, some small molecules cause non-specific allergies, typically histamine, and some molecules present may be toxic (e.g., melittin in the bee venom).

Therefore, procedures for obtaining, purifying and characterizing allergenic preparations that are suitable for biological testing are quite complex and involve combinations of preparative and analytical separation steps with immunochemical and biological tests and with measurements permitting the establishment of the chemical structure and conformation. Typically, such a procedure involves the extraction of a natural material with water, aqueous buffers (e.g., hydrogencarbonate or phosphate) or aqueous salt solutions (e.g., sodium chloride), followed by dialysis to remove low-molecular components and then by lyophilization. A precipitation step with ammonium sulfate is often included. The extraction should be rapid, to avoid enzymatic degradation. Preparative chromatographic steps (e.g., SEC, IEC) then produce a sufficient amount of material for finer, analytical separations (combina-

tions of SEC, RPC, HPAC, CE, etc.) into fractions whose biological activity is tested and whose chemical characteristics are studied. SEC is very useful for determining the molecular masses of the components. All these steps use a great variety of immunochemical techniques to improve the resolution and identification power [9].

Of course, these preparative techniques are sensitive to changes in the natural materials caused by environmental effects (pollen), degree of maturity (molds), etc. Furthermore, important allergens may be lost during the extraction due to the activity of proteases that are coextracted. Therefore, the production of pure allergens by recombinant DNA/RNA technologies has recently become an important alternative to the preparation from natural sources; it is a powerful tool in the characterization of allergens and will definitely be very important clinically in the near future [10,11].

In the following section, selected immunochemical methods are very briefly surveyed; their applications in combination with chromatographic and electromigration techniques are dealt with in some more detail in the appropriate sections on the separation procedures. Throughout the text, emphasis is placed on the most recent literature; many more references can be found in the works cited.

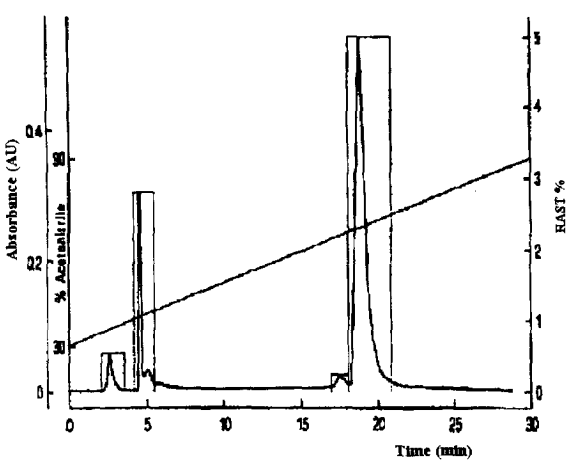

Fig. 1. RPC analysis of a fraction of methanolic extract of *Parietaria judaica* pre-separated by SEC and followed by RAST inhibition [19]. Column: LiChrosorb RP-C18; mobile phase: a gradient of ACN in 0.1% TFA.

2. Summary of immunochemical methods of analysis of allergenic preparations

There are two principal classes of methods for the study of biological activity of allergenic preparations, namely, in vivo and in vitro methods [3–6]. The former group is primarily based on skin tests on sets of patients which lead to expression of the allergic activity in terms of conventional units, e.g., BU (biological units), AU (allergy units) or HEP (histamine equivalent potency) [3,6,12,13]; this group is outside the scope of this review.

The latter class comprises a number of immunochemical methods that are based on the reactions of allergens (antigens, Ag) with specific IgE antibodies (Ab) contained in the sera of patients, with the formation of AgAb complexes that are then determined in various ways, often with the use of chromatographic or electrophoretic separations. Labelling with radioactive isotopes is used very often (radioimmunoassay, RIA) and the antibodies are frequently fixed at the surface of a solid support (solid-phase (radio)immunoassay, SPIA, SPRIA). The application of these methods then depends on the nature of the antibody used.

(A) A serum pool containing IgE prepared from the sera of a certain number of patients who exhibit a high sensitivity toward the allergen studied is used in one of the most common SPIA techniques, namely, the radio-allergosorbent test (RAST) (e.g. [14,15]). As usually performed, RAST is a diagnostic test for the presence of allergen-specific IgE antibodies and can also be used to quantitate single allergens or the total allergenic activity of a preparation. There are two principal modifications of the technique, direct RAST and the RAST inhibition (e.g. [15,16]). In direct RAST, the allergen preparation is immobilized, usually on CNBr-activated paper disks, and its interaction with the serum pool labelled with ^{125}I is then evaluated. In the inhibition test, the studied allergen solution is first incubated with the labelled serum pool and then with paper disks containing a standard allergen; the studied allergenic preparation then acts as the inhibitor of the interaction of the IgE-containing serum with the standard allergen. RAST is often combined with chromatographic separation, by measuring the biological activity of fractions obtained by HPLC (e.g., [17–19], Fig. 1);

however, a limited sensitivity of RAST after IEC has been reported [17].

(B) When an allergen has been successfully isolated, it is then possible to obtain the appropriate antibody and determine the allergen amount, using, for example, a polyclonal antibody-based radial immunodiffusion assay in an agarose gel. In the double-diffusion experiment, the antibody is placed in a central well and a serial dilution of the antigen forms a circle around the well producing precipitation lines. This method has, for example, been used to standardize ragweed extracts [20] and the allergens from domestic dust mites [21].

The immunoblotting technique is used for the specific identification of allergens. Components separated by gel electrophoresis are transferred to a nitrocellulose membrane (blotting) and the membrane with the blots is incubated in the presence of specific antibodies, e.g., Bermuda grass pollen [22–24], or an allergic serum pool, e.g., *Cocos nucifera* pollen [25]. The allergen–antibody complex is then detected using the corresponding antiIgE or antiIgG that is either labelled with ^{125}I, or conjugated with horseradish peroxidase or alkaline phosphatase. The blotting technique can not only detect the allergen–antibody complex, but other allergen properties can also be used for its detection, characterization and separation; e.g., the saccharide moiety in Bermuda grass pollen allergens and the role of saccharides in antigenicity have been studied using this technique and biotinylated lectins [22].

A very similar approach is used in the enzyme-linked immunosorbent assay (ELISA), where the allergen is immobilized in polystyrene wells; quantitative results can be obtained [22,25].

Immunoelectrophoretic techniques and affinity chromatography and electrophoresis are dealt with below, in the separation sections.

3. Physico-chemical properties of typical allergens from the point of view of their separation

Most allergens are proteins or glycoproteins and thus the separation conditions can generally be obtained from the extensive literature on protein separations. There are two principal properties which should always be borne in mind. First, the large protein molecules have many active sites of several kinds available for interactions in separation systems, which makes the separation mechanism complicated and causes pronounced dependences of the retention on even small changes in the experimental conditions. Second, proteins are easily denatured during separations, which places high demands on the separation system and on the speed of separation.

Some of the properties of proteins depends on the properties of their constituent amino acid residues, i.e. on the strength of the acid/base groups present and on the degree of hydrophobicity/hydrophilicity of the amino acid side chains. Therefore, proteins exhibit isoelectric points and their retention is strongly dependent on the pH. Other important contributions come from the primary, secondary, tertiary and quaternary structures, the importance of the higher structures increasing with increasing size of the molecule.

The chromatographic behaviour of proteins is generally much more complicated than their electrophoretic behaviour. RPC is the most versatile mode of chromatography for protein separations, as it utilizes both the acid/base and hydrophobic/hydrophilic properties of the solutes. Problems sometimes arise with the denaturing of proteins, caused by low pH values and high organic modifier contents in mobile phases used with the most common C_{18} stationary phases that are highly hydrophobic and are densely covered with the ligand; this difficulty is alleviated by using C_1 to C_4 stationary phases. Owing to the presence of many active sites in the solutes, the retention dependence on the mobile phase composition (primarily the pH, the content of the organic modifier and the ionic strength) is very steep and thus gradient elution must usually be employed.

The classical IEC procedures are applicable, but the separation efficiencies are usually somewhat poorer than in RPC and the solutes may be sorbed too strongly, again because of the presence of many active sites in the solute molecules. SEC is very useful in preseparations and permits quite reliable determination of molecular masses of proteins, provided that the system is carefully calibrated with standard mixtures and the experimental conditions are properly selected to suppress ion-exchange effects.

Problems of the solute denaturation are often

avoided when using hydrophobic-interaction chromatography (HIC) which employs milder conditions than RPC (moderate hydrophobicity and low ligand density of the stationary phase and aqueous mobile phases with high ionic strength values). Very high selectivities are exhibited in affinity separations, both chromatographic and electrophoretic.

A good summary of protein chromatography can be found in Ref. [26]; a very recent discussion of stationary phases for the purpose is in Ref. [27]. Electrophoresis offers very high separation efficiencies and fast analyses; however, it is unsuitable for the initial separation steps as its sample capacity is very low. For further references on chromatography and electrophoresis of proteins see below.

4. High-performance separations in allergen isolation and analysis

As pointed out above, high-performance separations are preceded by preseparation and preconcentration steps in the complex procedure of the obtaining of allergenic preparations. Traditionally, low-pressure chromatography on soft gels has been used for the purpose. However, gel filtration is disadvantageous as it greatly dilutes the allergen extract and thus must be combined with IEC for preconcentration. As the classical column chromatographic techniques are tedious (the procedure takes one or two days) and their efficiency is poor, they are now generally being replaced by high-performance methods.

4.1. Chromatography

HPLC has recently become one of the most popular methods in the analysis of allergens, primarily because continuous progress in the stationary phase technology is bringing improvements in the separation efficiency and speed of analysis. Both porous and nonporous materials with a particle size from 2 to 15 μm are used and larger pore sizes are usually preferred, typically 300 Å, in order to facilitate the penetration of the large solute molecules into the pores. Pellicular packings consisting of fused-silica beads coated with a thin porous layer enable rapid mass transfer and thus also fast analyses; an alternative is the use of nonporous (molded) phases which, however, have a very low specific surface area and thus suffer from a low loadability. Very small, regular and uniform particles of the stationary phases used bring the separation efficiencies for large molecules to several thousand theoretical plates per column, while the time of analysis is maintained low (typically, 15 to 30 min). For a survey see Ref. [27].

4.1.1. Size-exclusion chromatography

SEC is applied to raw extracts, to identify and quantify the active components. The elution profiles are characteristic and can be used for fingerprinting of allergen extracts. The separation conditions are mild and thus the allergen proteins are maintained in their native form. Provided that the effects other than size exclusion are minimized, the elution volume or time is a linear function of the logarithm of the molecular mass of the solutes. Therefore, after a careful calibration with a series of standard proteins, the relative molecular masses of allergens can be found from their retention volumes. An example of a calibration dependence, for a TSK G2000 SW column, is given in Fig. 2 (in this case, the parameters of the equation, $t_R = b \times \log M_r + a$, are $b = 151$, $s_b =$

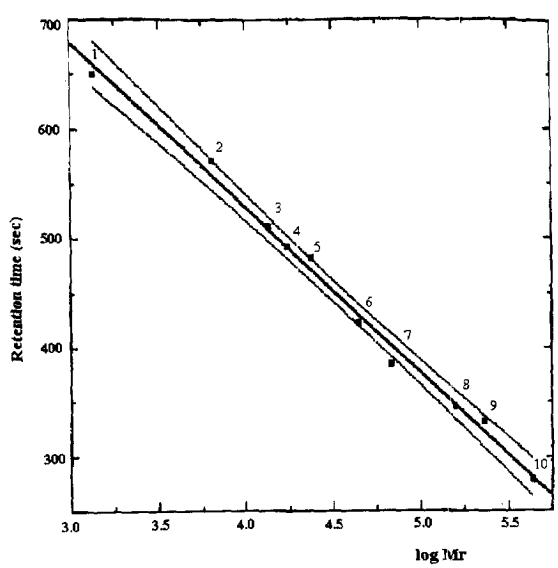

Fig. 2. Calibration dependence for a TSK G2000 SW SEC column; from [61] with permission. The dotted lines indicate the confidence interval at a significance level $\alpha = 0.05$; mobile phase: 0.2 *M* phosphate + 1 *M* KCl, pH 7.4.

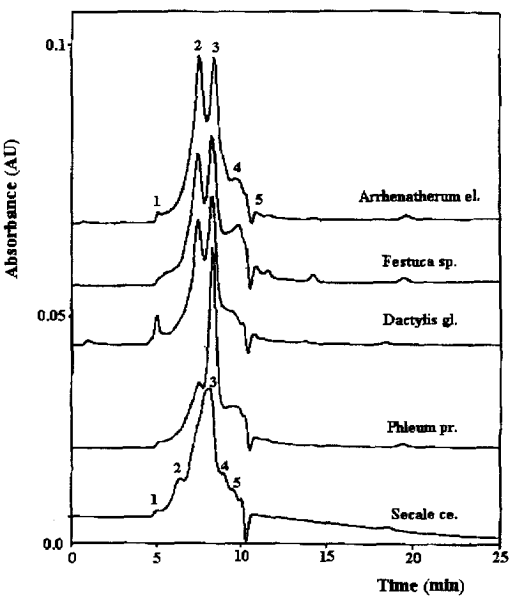

Fig. 3. SEC analysis of various pollen allergens; from Ref. [61] with permission. (For conditions see Fig. 2; the component labels correspond to those in Table 1.)

4.5; $a=1133$, $s_a=21$; the coefficient of correlation is $r=-0.997$) [28].

The reproducibility of the SEC retention time measurement has been studied [28–30], indicating a highly significant correlation between the molecular mass and the retention time ($p<0.05$) [29]; for allergens, the R.S.D. was lower than 0.5% [30]. Examples of separation of dialyzed and lyophilized pollens are given in Fig. 3 and the molecular masses of the main components are listed in Table 1 [28].

Linear retention vs. log of molecular mass dependences are obtained over a wide molecular mass range for synthetic polymer stationary phases; however, deviations from linearity may occur with proteins and glycoproteins owing to the varying shape of the solute molecules and their aggregation. A further cause of deviations from the linearity, i.e., the weakly basic and slightly hydrophobic properties of the stationary phase, can be largely compensated by judiciously selecting the mobile phase composition, primarily by working at the isoelectric point of the solutes; nevertheless, strongly basic solutes can still be irreversibly adsorbed. In order to evaluate non-ideal behaviour in SEC, a series of standard polymeric peptides [31] and standard proteins in guanidium hydrochloride media [32] have been devised.

Table 1
Estimated relative molecular masses M_r of pollen allergens 10^{-3} rel. mol. mass units [61].

Comp.No	*Arrhen. ru.*	*Dactyl. gl.*	*Secale ce.*	*Festuca* sp.	*Phleum pr.*
1	310	330	320	-	-
2	33	35	24	35	34
3	15	17	16	17	17
4	5	5.3	9	3.7	5
5	2	2.5	4	1.8	2.5

The effect of the column material on the allergenic activity and on the allergen patterns has further been studied [29], finding out by using RAST inhibition that the Bio-Sil TSK 250 is inert and exhibits no irreversible retention of allergens; the antigen/allergen patterns have also been unchanged.

Soft dextrane- or agarose-based cross-linked gels, employed in the classical gel chromatography, are useless in high-performance SEC because of their compressibility. Therefore, inorganic supports or those of organic polymers are employed, together with aqueous mobile phases. The standard silica supports are rigid, exhibit a well-defined pore distribution and tolerate a variety of mobile phases, except those with a pH greater than ca. 8. However, ionic and hydrophobic interactions of proteins with the stationary phase may occur depending on the ligand bonded and the number and distribution of the unreacted silanol groups, leading to errors in the molecular mass determined, lowered recoveries and poorer biological activity.

Polymeric hydrophilic stationary phases, such as TSK G2000 or 3000 SW or HEMA Bio materials, in combination with phosphate or acetate buffer mobile phases containing sodium chloride, are commonly used to purify and characterize allergens (see, e.g., [18,19,28,30,34,35]. Doubly distilled water containing 0.005 *M* sodium azide, combined with two columns, TSK 2000 SW and TSK 3000 SW, has been used to purify and fingerprint Parietaria allergenic extracts [35]. On the other hand, the mobile phase of distilled water combined with the silica-based stationary phase Protein Pak 125 has yielded a poor resolution of a Lolium perenne crude extract [36]. An ionic strength similar to the physiological

conditions (0.15 *M* phosphate-buffered saline) has been recommended to avoid aggregation of allergen molecules and to minimize ionic interactions between glycoproteins and a silica-based stationary phase. The method has been applied to the purification of Parietaria judaica [37] and fungal [7] allergens. Polymerization of a glycoprotein allergen from *P. judaica* has been observed on storage of the dialyzed and lyophilized material in solution; the polymer dissociated on addition of urea [38].

A distilled water mobile phase and a Protein Pak 125 column have been employed to separate a contaminant component from the olive pollen allergen [39]; the contaminant exhibited a longer retention due to non-specific hydrophobic interactions. A hydrophilic polyester packing, Shodex OHpak B-804/S, with 0.02 *M* ammonium acetate, pH 4.75, has been used for the characterization of insect venoms [40]; the components of the bee, wasp and hornet venoms have been satisfactorily resolved, but basic peptides have been strongly adsorbed. A SigmaChrom GFC-1300 polysaccharide-based gel filtration column and a 100 m*M* phosphate mobile phase, pH 6.8, permitted a better separation of the Oxyuranus scutellatus canni venom than a hydrophilic bonded silica [41].

The SEC method is fast and reproducible and thus can be used for process control [42]. The improvement in the resolution, the speed of analysis and the acceptable sample size is demonstrated in Fig. 4 where SEC of Dactylic gl. on a TSK G2000 SW column is compared with the classical gel filtration on Sephadex G-100 [43]. The high speed analysis minimizes decomposition of labile allergens and the high recovery, 80 to 98%, makes it suitable for industrial-scale processes. A disadvantage of SEC is a somewhat lower separation efficiency compared with RPC.

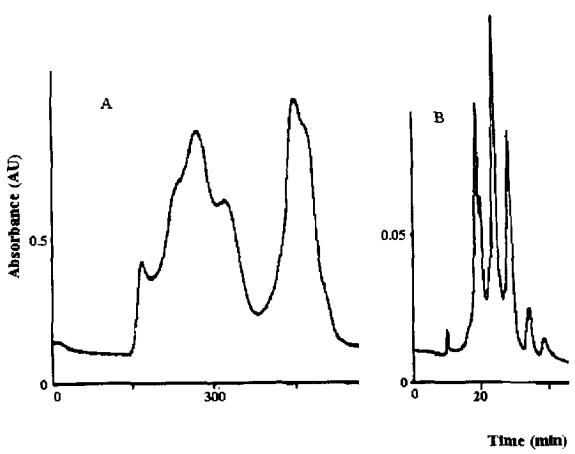

Fig. 4. Comparison of chromatograms of pollen extract from *Dactylis gl.* on (A) a TSK G2000 SW and (B) a Sephadex G-100 column [43]. Mobile phase: 0.1 *M* phosphate buffer +0.02% w/v sodium azide, pH 7.0; amounts injected: (A) 300 μg, (B) 25.7 mg.

4.1.2. Ion-exchange chromatography

IEC is well suited for allergen analysis provided that the mobile phase pH permits dissociation of the acid/base groups on the allergen molecule, i.e., it is at least one pH unit below p*I* (−log isoelectric point) for cation-exchange or one pH unit above p*I* for anion-exchange [44]. The separation is influenced by the pH, the nature of displacing salt and the ionic strength. The buffer concentrations usually vary between 10 and 100 m*M*. Volatile buffers, such as triethylammonium bicarbonate or ammonium acetate, are employed to facilitate lyophilization. In view of the steep retention dependence on the experimental conditions (see Section 3), gradient elution is commonly used (a gradient of pH or of ionic strength – to 1 *M* monovalent salt or 0.5 *M* bivalent salt). The mobile phases usually contain further components, e.g., stabilizers, denaturing agents, surfactants, etc. Additives are used to alter the selectivity, decrease the retention or increase the recovery by stabilizing the proteins. Chelating agents prevent contamination of isolated allergens by metals; reducing agents, such as dithiothreitol or β-mercapto-ethanol, are added to retard oxidation of labile proteins; surfactants improve the solubility of proteins. A subambient temperature is necessary for work with very labile proteins.

The molecular mass and shape of allergen molecules determine the pore diameter of porous ion-exchangers which must be sufficiently large to admit the allergen molecules (i.e. 300 Å for allergens of mass of 50 to 100 kDa).

High-speed IEC of proteins is enabled by the use of monodisperse nonporous stationary phase particles, due to enhanced mass transport [45]. A weak anion exchanger consisting of a polymeric core with a polyethyleneimine surface enables a separation of

four proteins within 24 s, with a high recovery for small samples (82% with a 5 μg sample).

Both cation (Mono S HR 5/5) and anion (Mono Q HR 5/5) exchangers have been tested for Timothy pollen [34], the best results being obtained with gradient elution using 0.5 *M* NaCl in 10 m*M* HEPES–NaOH buffer, pH 8.0; broad solute peaks have been caused by the presence of glycoisoforms of the principal allergen. Other examples are the use of the Mono Q HR 10/10 anion exchanger and Mono S cation exchanger with a step elution with the TRIS buffer and NaCl for the Der pI and Der fI allergens from *Dermatophagoides pteronyssinus* [46] and *Dermatophagoides farinae* [47], respectively. A DEAE-5PW anion-exchange column with a linear ionic strength gradient (sodium acetate in the TRIS buffer) and RAST inhibition has been used for allergen preparation from *P. judaica* [48] (Fig. 5). A homopolymerization of the protein has been observed; an increase in the ionic strength during the chromatographic run has led to dissociation of the oligomers [48]. A combination of SEC and IEC has been useful in the purification and identification of *P. judaica* pollen extracts [49].

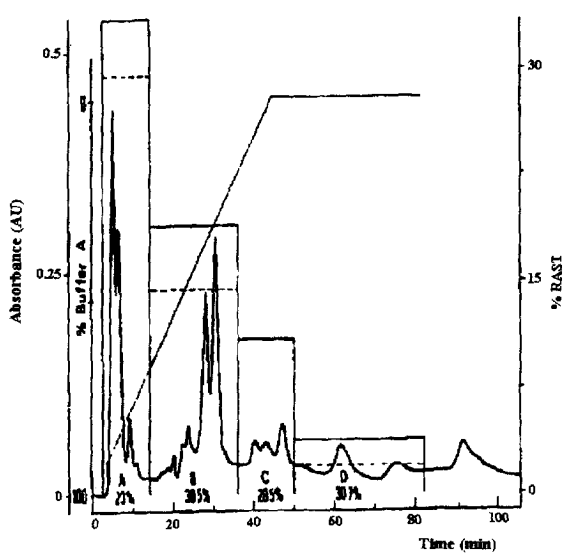

Fig. 5. IEC separation of *Parietaria judaica* pollen extract followed by RAST inhibition of the fractions before dialysis (solid line) and after it (dashed line) [48]. Column: DEAE-5PW; mobile phase: a gradient of sodium acetate (500 m*M*) in 10 m*M* Tris–acetic acid buffer (pH 7.0)

Anion-exchange HPLC is a suitable method for the determination of the carbohydrate composition of glycoproteins. A glycoprotein is first hydrolyzed by an acid (TFA or HCl), evaporated to dryness to remove the acid and then analyzed [50].

Ion-exchange is also employed in chromatofocusing; a pH gradient is generated in the column, the proteins are desorbed at pH values lower or equal to their isoelectric points and readsorbed at higher pH values, the proteins with identical isoelectric points focusing together.

4.1.3. Reversed-phase chromatography

As pointed out above, RPC is one of the most common methods used in the separation of peptides and proteins which are not very large; silica-based stationary phases are primarily used [27]. To prevent denaturing of allergens, short-chain chemically bonded phases, C1 to C4, are preferable, with mobile phases containing low percentages of organic modifiers. The separation is then primarily governed by the (glyco)protein structure and hydrophobicity.

The RPC separations of allergens are generally faster and more efficient than SEC ones owing to more rapid mass transport; furthermore, the isolated allergens have a higher purity. Very rapid separations have been attained when using stationary phases with a pore diameter of 300 Å [51].

The elution with a gradient of acetonitrile in aqueous TFA is very common. An example is the separation of the allergens from the bee venom [33]. In this case, silica-based stationary phases are unsuitable because of irreversible adsorption of the strongly basic polypeptide melittin that accounts for 50% of the bee venom. Therefore, the polymeric HEMA Bio C_{18} phase has been used for both analytical and preparative purposes (Fig. 6). The procedure permitted an isolation of 3.2 mg of the pure main allergen, phospholipase A_2, from an injected amount of 2 ml of a solution containing 20 mg of bee venom; the phospholipase A_2 retained its full biological activity.

The fractions separated by RPC are often analyzed by means of RAST inhibition for their allergenic activity (see the RPC-RAST of *P. Judaica* [17], Fig. 1 above); immunoblotting can also be used for this purpose [52]. RPC is also very useful for quantitative estimation of the allergen contents in the extracts; the various allergens are primarily differentiated in terms

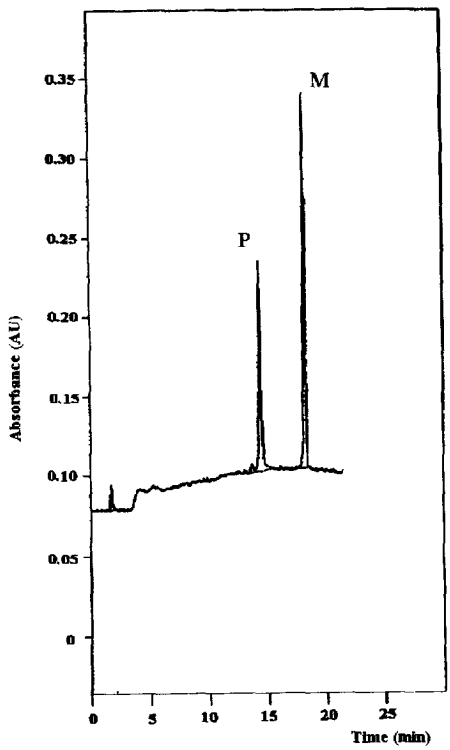

Fig. 6. RPC analysis of bee venom [33]. Column: HEMA-Bio 1000 C18; mobile phase: a gradient of ACN in 0.2% TFA; P-phospholipase A_2, M-melittin.

of their hydrophobicity/hydrophilicity. For example, hydrophilic glycoproteins with molecular masses of 33 to 34 kDa and more hydrophobic proteins with molecular masses of 26 to 33 kDa, which are components of allergen preparations from the pollen of *Dactylis glomerata*, *Festuca pratensis*, *Holcus lanatus*, *Poa pratensis*, *Lolium perenne* and *Phleum pratense*, can be separated on a Vydac C4 column with an acetonitrile gradient in aqueous TFA. The *Phleum pratense* allergen Phl p 5 can be separated into 5a and 5b subgroups [52] (Fig. 7). Under the same conditions, a broad peak of the mite allergen Der pI was observed; the width of the peak can be caused either by C-terminal variations in the protein (differences in the chain length or in the amino acid content), or by glycosylation [46].

RPC is the most suitable method for the separation of glycoproteins [53]. Most glycoproteins are multiglycosylated, i.e., contain oligosaccharides at two or more positions in the amino acid sequence. The

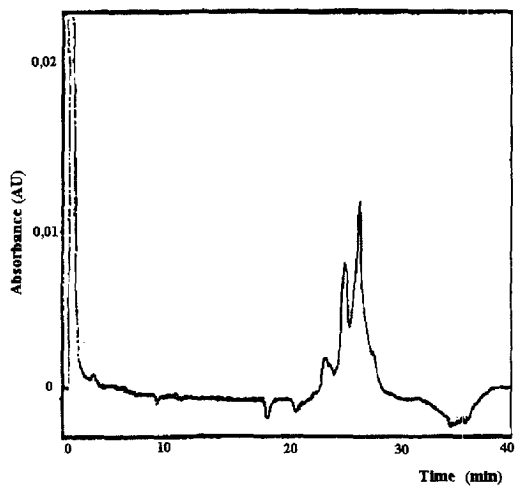

Fig. 7. RPC separation of *Phleum pratense*, Phl p 5 (A) and *Lolium perenne*, Lol p 5 (B), into two isoforms, Phl p 5a and 5b and Lol p 5a and 5b, respectively; from Ref. [52] with permission. Column: Vydac C4 214 TP 52; mobile phase: a gradient of ACN in 0.1% TFA.

most difficult task is the separation of proteins which have an identical amino acid sequence but differ in the number of bound oligosaccharides and/or their structure. Using a gradient of isopropanol from 0 to 30% in 0.1% aqueous TFA in the separation of a tryptic digest, these compounds can be differentiated [53].

A simple and cheap alternative to HPLC in separations of low-molecular mass components of allergenic preparations, e.g., pentoses and hexoses, is high-performance thin-layer chromatography (HPTLC) [54].

4.1.4. Affinity chromatography

Affinity chromatography separates components of mixtures on the basis of biospecific interactions between the components and a ligand (affinant) immobilized on a support. The specifically sorbed substances are eluted from the column by an agent that dissociates the solute–ligand complex. The classical, low-pressure affinity chromatography is at present largely replaced by high-performance techniques employing rigid supports with chemically-bonded affinants which permit faster, more efficient separations and more sensitive quantitation. Success in a separation depends on the choice of the affinant,

the inert support, the method of affinant immobilization and the chromatographic conditions.

Two affinant types are employed, those specific for a particular molecule and group-specific ones. They must satisfy a number of conditions, namely, the solute binding must be reversible, the affinants must be chemically stable and they must have suitable properties for binding to the support. The conditions of desorption of specifically bound solutes are important for the recovery of the solutes and their biological activity. Specific desorption agents compete with the immobilized affinant for the solute binding sites and usually permit the obtaining of non-denatured solute molecules. Non-specific desorption agents are used when a specific one is not available or when the affinant–solution interaction is very strong; they involve aqueous solutions of a high or a low pH or of a high ionic strength and chaotropic agents. In the latter case, the solutes recovered are often denatured. For a survey of these techniques see Refs. [55–57].

Affinity HPLC using immobilized monoclonal antibodies as affinants has been used to isolate and purify certain allergens. A *Parietaria judaica* pollen extract has been separated into several fractions by elution with a 2 *M* NaCl solution and almost all the fractions have been RAST positive [17,58]. The major peanut allergen has been purified in this way [59]. Classical chromatography has also been employed for the purification of the antigen BG-60 of Bermuda grass pollen using a Blue-Gel column and a stepwise elution with an NaCl solution [22], or for isolation of grass pollen allergens on a Concanavalin A-Sepharose [60].

4.2. Electromigration

Electrophoretic methods have become extremely popular lately, mainly because of the availability of reliable instrumentation for capillary techniques. Nevertheless, slab gel electrophoresis has long been a very important tool in the isolation, purification and analysis of allergens. The principal attractive features of capillary electrophoresis are its simplicity and rapidity of operation, the very high separation efficiencies attained, the simple optimization of the separation conditions and a relatively low cost of analysis.

Capillary electrophoresis now offers a variety of specific techniques, such as zone electrophoresis, isotachophoresis, isoelectric focusing, hybrids between electrophoresis and HPLC (e.g., micellar electrokinetic chromatography and electrochromatography) and gel capillary electrophoresis. Moreover, it is very often combined with immunochemical and affinity principles. In view of very low sample capacities, electrophoretic techniques are generally unsuitable for preparative purposes.

4.2.1. Capillary electrophoresis

The main problem in the application of capillary electrophoresis to separations of allergens, i.e., mostly proteins and glycoproteins, is the solute adsorption on the capillary walls leading to peak tailing and thus to deterioration in the separation efficiency and in the reliability of both the qualitative and quantitative data. There are several ways to deal with this difficulty:

(a) The pH of the running buffer is kept very low, less than 2. The capillary walls then become positively charged as the surface silanols are protonated and the positively charged proteins are repulsed from the walls. An example of such a separation is given in Fig. 8 [33] where bee venom components are resolved in 150 m*M* phosphoric acid, pH 1.8. The two main components, phospholipase A2 and melittin, have been identified on the basis of the peak matching with those of the standard solutes. A very high efficiency of this separation is demonstrated by the fact that the phospholipase A_2 standard compound (Sigma), which appeared to be homogeneous in a RPC separation, has been split to at least three components, the content of the main component being only ca. 85%. The procedure is reproducible (Table 2) and is now routinely used for checking the quality and stability of bee venom allergen preparations. The same running buffer has also been used to analyze wasp, pollen, mite, canine and feline allergens [28,30,61].

(b) Inorganic salts or surfactants are added to the running buffer, e.g., α,ω-bis-quaternary ammonium alkanes [62,63]. An optimal separation of glycoproteins then occurred at a pH of 7 to 9, the glycoforms migrated in order of increasing number of sialic acids in the molecule [62].

(c) The capillary walls are physico-chemically

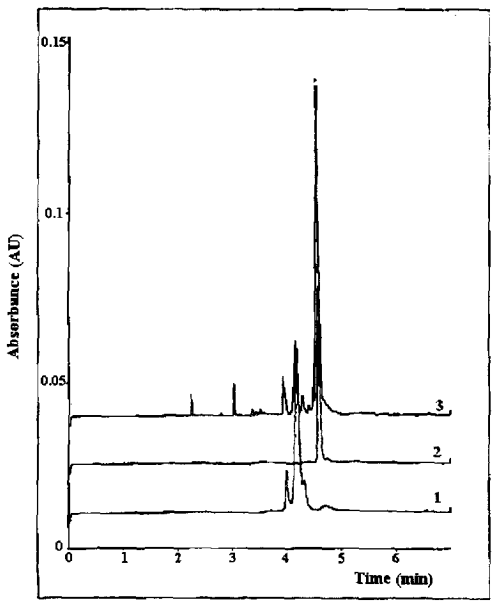

Fig. 8. CE analysis of bee venom components [33]. 1-Phospholipase A_2 (Sigma), 2-melittin (Sigma), 3-bee venom. Electrolyte: 0.15 *M* phosphoric acid, pH 1.8; capillary: I.D. 75 μm, L_T=75 cm, L_D=60 cm; voltage, 20 kV.

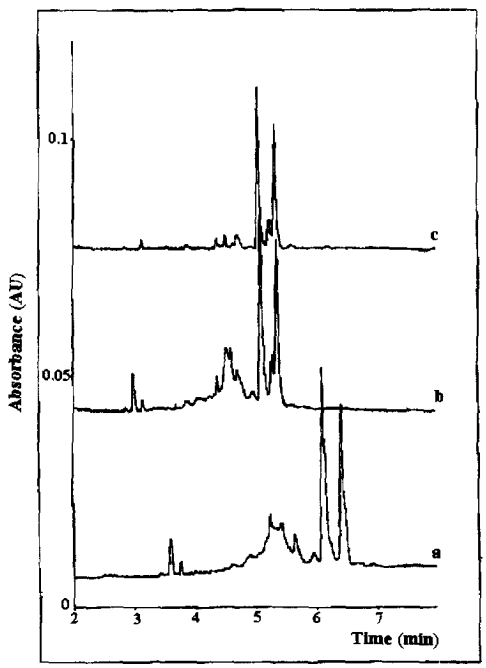

Fig. 9. Effect of the capillary wall coating on the separation of raw extract of *Phleum pratense* (a,b) and its low molecular mass fraction (c) [61]. (a) Uncoated capillary, (b,c) HEMA coated capillary. Experimental conditions as in Fig. 8, except for L_T=62 cm, L_D=47 cm.

modified. For example, the separation of the glycoprotein allergens from *Phleum pratense* is improved when the capillary walls are coated with a film of the hydroxyethylmethacrylate (HEMA) polymer (Fig. 9) [61]. Not only that the peak shape is improved, but also the elution times are substantially decreased. Chemical modification of capillaries with vinylpyrrolidone and vinylimidazole and the effect of

Table 2
Comparison of the RPC and CE analytical parameters for the analysis of bee venom components [33]

Analytical parameter	RPC		CE	
	Phospholipase A_2	Melittin	Phospholipase A_2	Melittin
R.S.D.				
Peak area	5.2	2.8	2.1	1.5
Retention time	1.4	1.0	6.5	5.6
Detection limit				
ng	56	30	0.4	0.15
μg/ml	5.6	3	4.5	1.6
Linear dynamic range (orders of concentration)	3		2	
Analysis time	45		6	

Experimental conditions: RPC-Column, HEMA-Bio 1000 C-18, 10 μm, (150×3.3 mm I.D.); mobile phase: a linear gradient from 0 to 50% of ACN in 0.2% TFA in 20 min, followed by a rise to 100% in 20 min; temp. 37°C; flow-rate 0.5 ml/min; UV detection at 215 nm; CE-electrolyte, 150 m*M* phosphoric acid, pH 1.8; capillary L_C=75 cm, L_D=60 cm, I.D.=75 μm; potential, 20 kV; UV detection at 190 nm.

the modification on separations of basic proteins were also recently studied [64].

Capillary electrophoresis has also been found useful in determining the number and distribution of oligosaccharide linkage positions in *O*-linked glycoproteins [65].

Neutral species can readily be separated by micellar electrokinetic chromatography (MEKC) where surfactants are added to the running buffer at concentrations exceeding the critical micelle concentration (CMC). The micelles form a pseudostationary phase with which the solutes interact through hydrophobic and electrostatic interactions and migrate either with or against the electroosmotic flow, depending on the micelle charge polarity; the separation of neutral solutes then depends on the selectivity of their partitioning between the buffer and the micelle.

A system of a borate running buffer containing sodium dodecylsulfate (SDS, whose CMC is 8 to 9 mmol/l) has been successfully applied to separations of lyophilized allergens [28,30,61]. An example of a MEKC separation is the analysis of *Phleum pratense* WHO international standard [30] (Fig. 10). The

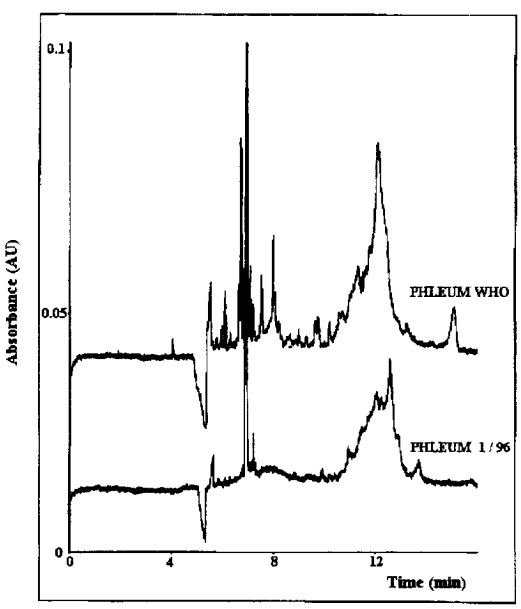

Fig. 10. Comparison of MEKC analyses of *Phleum pratense* WHO international standard and SEVAC standard I/96 [30]. Electrolyte: 20 m*M* SDS+50 m*M* borate, pH 9.3; capillary: L_T=75 cm, L_D=60 cm; voltage: 20 kV.

oligosaccharides cleaved from glycoproteins by the endoglycosidase digestion have been analyzed after derivatization with 2-aminoantipyrine, using a system of 0.1 *M* phosphate buffer containing 50 m*M* tetrabutylammonium bromide at pH 5.0 [66].

Isotachophoresis has somewhat lost its importance recently, in favour of capillary zone electrophoresis. Nevertheless, it has been recommended for characterization (fingerprinting) of allergen extracts during their manufacture and quality control [67]. The time of analysis is 15 to 20 min with a leading electrolyte of 0.01 *M* chloride buffered with Tris (pH 8.2) plus 0.2% w/v hydroxyethylcellulose and a terminating electrolyte of β-alanine buffered to pH 10 with barium hydroxide.

4.2.2. Gel and capillary gel electrophoresis

Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was introduced some time ago [68] as a very useful slab gel technique and is still often used to characterize allergen extracts in terms of their molecular masses. On heating proteins with SDS in the presence of the mercaptoethanol reducing agent, the disulfide bridges are broken and SDS is bound to dissociated protein chains, the SDS–polypeptide complexes having a random coil configuration. One gram of any protein binds 1.4 g of SDS and thus similar mass-to-charge ratios (similar electrophoretic mobilities) are obtained. Proteins are separated by sieving through the gel matrix according to their size (molecular mass); this holds for proteins that exhibit no post-translational modification and whose molecular masses are in a range of 10 to 200 thousand rel. mol. mass units. With smaller proteins, the intrinsic charge on the amino acid chains may affect the overall charge of the SDS–polypeptide complexes. Post-translational modifications cause changes in both the mass and the charge; for example, glycosylation leads to an increase in the molecular mass whereas phosphorylation and sulfonation increase the charge.

The application of gel electrophoresis, i.e., the native PAGE, SDS–PAGE, IEF and two-dimensional electrophoresis, to the identification of plant varieties has recently been reviewed [69]. Identification of electrophoretically separated proteins and interfaces between two-dimensional PAGE (1st dimension, IEF, 2nd dimension, SDS–PAGE) and a specialized

technique of amino acid sequencing and mass spectrometry have also been generally discussed [70]. Proteins can be identified through antibody recognition (immunoblotting), or by peptide mapping or direct amino acid sequencing. Gel separated proteins can further be analyzed using MALDI–TOF–MS. The advantages of the latter procedure are a high accuracy, sensitivity and sometimes also tolerance toward buffers.

Capillary gel electrophoresis (CGE), introduced in the eighties [71,72] and recently reviewed by Guttman [73], brings a number of advantages over slab gel technique. The separation is about three times faster and more than five times more efficient (more than 10 million plates), the resolution is substantially improved, quantitation is accurate and the procedure is readily automated. Standard CZE capillaries are employed and filled with either chemical or physical gels. Chemical gels are cross-linked and their pore structure, size and thus also the solute electrophoretic mobilities are determined by the concentrations of the monomer (5 to 15%) and the cross-linking agent (0.1 to 0.3%); polyacrylamide gels are most common. The drawbacks of chemical gels involve sensitivity to heat, a danger of bubble formation and easy damage to the gel on injection of impure samples. Physical gels are non cross-linked, linear or slightly branched polymer networks that are flexible and can be readily replaced.

The solute electrophoretic mobilities depend on the sizes and shapes of the molecules and the gel pores; however, the migration rate may be affected by adsorption and ion-exchange phenomena. CGE exhibits a great potential for allergen characterization, especially the capillary SDS–PAGE mode for determination of molecular masses. The SDS-protein complexes are detected at the anode, as they are negatively charged; the electroosmotic flow is eliminated because the gel is bound to the capillary wall.

The separation mechanism can be checked by using the Ferguson plots in which the log mobilities are plotted against %T for individual proteins. The straight lines are extrapolated to 0%T; the intercept then yields the mobility in free solution (in the absence of the gel) and coincidence of the intercepts indicates that there is no separation without the gel and thus the separation mechanism is solely based on the differences in the molecular mass. The Ferguson plot slopes are proportional to the molecular mass [74].

4.2.3. Affinity electrophoresis and immunoelectrophoresis

To our knowledge, affinity capillary electrophoresis has not yet been applied to allergen separations. Nevertheless, there is a potential in this technique, using either specific antibodies as the affinants, or employing other properties of allergens, e.g., separating glycoprotein allergens with lectins as affinants. To develop such techniques, experience obtained in the separation of other large molecules can be used (for a review of various types of affinity electrophoresis separations see Refs. [74–79].

In affinity electrophoresis, the affinant is either bound to a gel and the procedure is analogous to normal capillary gel electrophoresis, or free affinity capillary electrophoresis is carried out, with the affinant present in the running buffer as well as in the sample.

Immunoelectrophoretic techniques combine the high resolving power of electrophoretic separations and the selectivity of immunochemical methods. Of all the immunochemical approaches available, the following have been successfully applied to characterization and analysis of allergens:

(a) Rocket immunoelectrophoresis is suitable for the estimation of allergen concentration. Different amounts of the antigen migrate electrophoretically into an agarose gel containing a specific antibody. A rocket shaped precipitate is formed and the height of the spot depends on the antigen amount. (b) Classical immunoelectrophoresis separates proteins in antibody-free agarose gel. The protein bands then diffuse into agarose gel containing antibodies and precipitation lines are formed. (c) Crossed immunoelectrophoresis is often used to study antigenic heterogeneity of allergenic extracts. The components are first separated in agarose gel in one direction, then the separated bands electrophoretically migrate into gel containing an antibody in the other direction. In this way, antigenicity of birch pollen isoallergens [80] and of those from *Cocos nucifera* pollen [81] have been studied. (d) Fused rocket immunoelectrophoresis is useful when employed in combination with another separation method. The separated fractions diffuse into antibody-free agarose gel and

then electromigrate into an antibody-containing gel. The precipitation lines formed reveal the heterogeneity of the separated fractions. This method has been used, for example, to characterize birch pollen isoallergens [80].

5. Conclusion

The field of isolation, characterization and analysis of allergens is highly complex and judicious combinations of approaches and experimental techniques are imperative. Useful information can be found in the Proceedings of Paul-Ehrlich Seminars [82]. Critical comparison of various methods is very useful as a guide in the selection of most suitable methods for particular purposes. As an example, a comparison of the analytical parameters of RPC and CE is given in Table 2 above for the analysis of the bee venom components [33]. It can be seen that the CE method is faster and its mass detection limits are lower than in RPC, whereas the concentration detection limits are similar for the two techniques. The RPC detection limit, calculated here for a 10 μl injection, can further be decreased when injecting a greater volume. The lower precision of the RPC peak area is partly caused by manual injection, the use of an autosampler would improve it. The poorer precision of the retention time measurement in CE is primarily due to instability of the electroosmotic flow. When very small amounts of the test material are available, then CE is advantageous because it requires extremely small sample amounts (units to tens of nl).

6. List of abbreviations

ACN	Acetonitrile
CE	Capillary electrophoresis
CGE	Capillary gel electrophoresis
CIE	Crossed immunoelectrophoresis
CMC	Critical micelle concentration
CZE	Capillary zone electrophoresis
HEPES	*N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid
ELISA	Enzyme-linked immunosorbent assay
FRIE	Fused rocket immunoelectrophoresis
HPAC	High-performance affinity chromatography
HPTLC	High-performance thin-layer chromatography
IE	Immunoelectrophoresis
IEC	Ion-exchange chromatography
IEF	Isoelectric focusing
ITP	Isotachophoresis
MEKC	Micellar electrokinetic chromatography
PAGE	Polyacrylamide gel electrophoresis
RAST	Radio-allergosorbent test
RIA	Radio-immunoassay
RIE	Rocket immunoelectrophoresis
RPC	Reversed-phase chromatography
SDS	Sodium dodecylsulfate
SDS–PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
SEC	Size-exclusion chromatography
SPIA	Solid-phase immunoassay
TFA	Trifluoroacetic acid

References

[1] R. Müller, Insektenstichallergie. Klinik, Diagnostik und Therapie. G. Fischer Verlag, Stuttgart 1988.
[2] H. Lowenstein, Techniques for definition and identification of allergenic molecules. Possible use for predicting optimal diagnostic and therapeutic extracts. in: C. Steffen, H. Ludwig (Eds.), Developments in Immunology, Vol. 14, Clinical Immunology and Allergology, Elsevier, Amsterdam 1981, pp. 265–274.
[3] J. Bousquet, B. Guérin, F.-B. Michel, Units of allergen extracts, in: R. Kurth (Ed.), Regulatory control and standardization of allergenic extracts, VI. Int. Paul-Ehrlich Seminar, Sept. 5–7, Frankfurt am Main, 1990, pp. 105–116.
[4] J.W. Yunginger, Ann. Allergy 66 (1991) 107.
[5] T.A.E. Platts-Mills, M.D. Chapman, J. Allergy Clin. Immunol. 87 (1991) 621.
[6] S. Dreborg, Allergy 66 (1993) 63.
[7] N. Rubio, A. Brieva, J. Chromatogr. 398 (1987) 366.
[8] S. Dreborg, R. Einarsson, Allergy 47 (1992) 418.
[9] V. Pacáková, K. Štulík, Chem. Listy 89 (1995) 354.
[10] O. Scheiner, H. Breiteneder, C. Dolocek, M. Duchéne, C. Ebner, F. Ferreira, K. Hoffmann, S. Schenk, R. Valenta, D. Kraft, Molecular and Functional Characterization of Allergens: Basic and Practical Aspects, Arbeiten aus dem Paul-Ehrlich Institut, Gustav Fischer Verlag, Stutgart, 1994, pp. 221.
[11] O. Scheiner, D. Kraft, Allergy 50 (1995) 384.
[12] P.C. Turkeltaub, in: E. Middleton Jr., E.F. Elliss, C.E. Reed, N.F. Adkinson Jr., J.W. Yunginger, (Eds.), Allergenic Extracts: In Vivo Standardization. Allergy, Principles and Practice, third ed., Mosby Co., St. Louis, MO, 1988, pp. 388–401.
[13] A. Basomba, Allergy 14 (1993) 71.

[14] J.M. Varga, M. Ceska, J. Allergy Clin. Immunol. 49 (1972) 274.
[15] G.J. Gleich, J.B. Larson, R.T. Jones, J. Baer, J. Allergy Clin. Immunol. 53 (1974) 158.
[16] L. Yman, G. Poterius, R. Band, Dev. Biol. Stand. 29 (1975) 151.
[17] E. Bolzacchini, P. DiGennaro, G. DiGregorio, B. Rindone, P. Falagiani, G. Mistrello, I.B. Sondergaard, J. Chromatogr. 542 (1991) 337.
[18] D.H. Calam, J. Davidson, A.W. Ford, J. Chromatogr. 266 (1983) 293.
[19] N. Rubio, A. Brieva, M. Rubio, J. Chromatogr. 392 (1987) 447.
[20] H. Baer, H. Godfrey, C.J. Maloncy, J. Allergy 45 (1970) 347.
[21] M.D. Chapman, P.W. Heymann, S.R. Wilkins, J. Allergy Clin. Immunol. 80 (1987) 184.
[22] S.N. Su, P. Shu, G.X. Lau, S.Y. Yang, S.W. Huang, Y.C. Lee, J. Allergy Clin. Immunol. 83 (1989) 1124.
[23] F. Mattiesen, M.J. Schumacher, H. Löwenstein, J. Allergy Clin. Immunol. 83 (1989) 1124.
[24] S.A. Ford, B.A. Baldo, J. Allergy Clin. Immunol. 29 (1987) 711.
[25] K.S. Jaggi, N. Arora, P.N. Niphadkar, S.V. Gangal, J. Allergy Clin. Immunol. 84 (1989) 378.
[26] C.T. Mant, R.S. Hodges (Eds.), High-Performance Liquid Chromatography of Peptides and Proteins: Separation, Analysis and Conformation, CRC Press, Boca Raton, Ann Arbor, Boston, London 1991.
[27] K. Štulík, V. Pacáková, J. Suchánková, H. Claessens, Anal. Chim. Acta in press.
[28] V. Pacáková, J. Pechancová, K. Štulík, J. Chromatogr. B 681 (1996) 47.
[29] R. Wahl, J.J. Maasch, W. Geissler, J. Chromatogr. 351 (1986) 39.
[30] El Hussen Arebi, MSc Thesis, Charles University, Prague 1996.
[31] C.T. Mant, R.S. Hodges, Requirements for Peptide Standards to Monitor Ideal and Non-Ideal Behaviour in Size-Exclusion Chromatography, in: C.T. Mant, R.S. Hodges (Eds.), High-Performance Liquid Chromatography of Peptides and Proteins: Separation, Analysis and Conformation, CRC Press, Boca Raton, Ann Arbor, Boston, London 1991, pp. 125–134.
[32] R. Nave, K. Weber, M. Potschka, J. Chromatogr. A 654 (1993) 229.
[33] V. Pacáková, K. Štulík, Pham Thi Hau, I. Jelínek, I. Vinš, D. Sýkora, J. Chromatogr. 700 (1995) 187.
[34] S. Haavik, B.S. Paulsen, J.K. Wold, J. Chromatogr. 321 (1985) 199.
[35] P. Falagiani, E. Cavallone, M. Nali, B. Rindone, S. Tollari, G. Crespi, J. Chromatogr. 328 (1985) 425.
[36] A. Brieva, N. Rubio, J. Chromatogr. 370 (1986) 165.
[37] N. Rubio, A. Brieva, J. Chromatogr. 407 (1987) 408.
[38] A. Bassoli, F. Chioccara, G. Di Gregorio, B. Rindone, S. Tollari, P. Falagiani, G. Riva, E. Bolzacchini, J. Chromatogr. 444 (1988) 209.
[39] N. Rubio, A. Brieva, B. Alcazar, J. Chromatogr. 403 (1987) 312.
[40] B. Renck, R. Einarsson, J. Chromatogr. 197 (1980) 278.
[41] R. Crouch, The Reporter (Supelco) 15 (1996) 5.
[42] R. Wahl, D. Meineke, H.J. Maasch, J. Chromatogr. 397 (1987) 307.
[43] D.H. Calam, J. Davidson, J. Chromatogr. 218 (1981) 581.
[44] M.P. Nowlan, K.M. Gooding, High-performance ion- exchange chromatography of proteins, in: C.T. Mant, R.S. Hodges (Eds.), High-Performance Liquid Chromatography of Peptides and Proteins: Separation, Analysis and Conformation, CRC Press, Boca Raton, Ann Arbor, Boston, London 1991, pp. 203–213.
[45] R.G. Hatch, J. Chromatogr. Sci. 31 (1993) 469.
[46] J.-P. Dandeu, J. Rabillon, M. Lux, B. David, J.-L. Guillaume, L. Camoin, J. Chromatogr. 599 (1992) 105.
[47] K.S. Mellbye, B.S. Paulsen, S. Dale, J. Chromatogr. 367 (1986) 247.
[48] I. Sondergaard, P. Falagiani, E. Bolzacchini, P. Civaroli, G. Di Gregorio, V. Madonini, A. Morelli, B. Rindone, J. Chromatogr. 512 (1990) 115.
[49] E. Bolzacchini, G. Di Gregorio, M. Nali, B. Rindone, P. Falagiani, G. Riva, G. Crespi, J. Chromatogr. 397 (1987) 299.
[50] Y.C. Lee, Anal. Biochem. 189 (1990) 62.
[51] W. Kopaciewicz, E. Kellard, G.B. Cox, J. Chromatogr. A 690 (1995) 9.
[52] B. Fahlbusch, W.-D. Müller, O. Cromwell, L. Jäger, J. Immunol. Methods 194 (1996) 27.
[53] F.W. Putnam, N. Takahashi, J. Chromatogr. 443 (1988) 267.
[54] R. Wahl, J.J. Maasch, W. Geissler, J. Chromatogr. 329 (1985) 153.
[55] T.M. Phillips, Theory and Practical Aspects of High-Performance Immunoaffinity Chromatography, in: C.T. Mant, R.S. Hodges (Eds.), High-Performance Liquid Chromatography of Peptides and Proteins: Separation, Analysis and Conformation, CRC Press, Boca Raton, Ann Arbor, Boston, London 1991, pp. 507–515.
[56] J. Turková, Bioaffinity Chromatography, J. Chromatogr. Library, Vol. 55, Elsevier, Amsterdam 1993.
[57] J.E. Van Eyk, C.Y. Mant, R.S. Hodges, High-Performance Affinity Chromatography of Peptides and Proteins, in: C.T. Mant, R.S. Hodges (Eds.), High-Performance Liquid Chromatography of Peptides and Proteins: Separation, Analysis and Conformation, CRC Press, Boca Raton, Ann Arbor, Boston, London 1991, pp. 479–491.
[58] E. Bolzacchini, P. DiGennaro, G. DiGregorio, B. Rindone, P. Falagiani, G. Mistrello, I.B. Sondergaard, J. Chromatogr. 548 (1991) 229.
[59] A.W. Burks, G. Cockrell, C. Connaughton, R.M. Hellar, J. Allergy Clin. Immunol. 93 (1994) 743.
[60] B. Karlstam, B. Nilson, J. Immunol. Methods 54 (1982) 119.
[61] V. Pacáková, J. Pechancová, K. Štulík, J. High Resol. Chromatogr. 18 (1995) 582.
[62] E. Watson, F. Yao, J. Chromatogr. 630 (1993) 442.
[63] R.P. Oda, B.J. Madden, T.C. Spelsberg, J.P. Landers, J. Chromatogr. A 680 (1994) 85.
[64] R.J. Xu, V. Vidal-Madjar, B. Sébille, J.C. Diez-Masa, J. Chromatogr. A 730 (1996) 289.

[65] P.L. Weber, C.J. Bramich, S.M. Lunte, J. Chromatogr. A 680 (1994) 225.
[66] W. Nashabeh, Z. El Rassi, J. Chromatogr. 536 (1991) 31.
[67] C.H.M.M. deBruijn, J.C. Reijenga, G.V.A. Aben, Th.P.E.M. Verheggen, F.M. Everaerts, J. Chromatogr. 320 (1985) 205.
[68] A. Saphiro, E. Vinuela, J. Maizel, Biochem. Biophys. Res. Commun 28 (1967) 815.
[69] R.J. Coole, J. Chromatogr. A 698 (1995) 281.
[70] S.D. Patterson, Anal. Biochem. 221 (1994) 1.
[71] S. Hjertén, J. Chromatogr. 270 (1983) 1.
[72] A.S. Cohen, B.L. Karger, J. Chromatogr. 397 (1987) 409.
[73] A. Guttman, Electrophoresis 17 (1996) 1333.
[74] B.D. Hanes, D. Rickwood (Eds.), Gel Electrophoresis of Proteins, IRL, Washington, 1983.
[75] V. Hořejší, M. Tichá, J. Chromatogr. 376 (1986) 49.
[76] K. Takeo, J. Chromatogr. A 698 (1995) 89.
[77] T.C. Bog-Hansen, in: W.H. Scouten (Ed.), Solid Phase Biochemistry, Analytical and Synthetic Aspects, J. Wiley, New York 1983, pp. 223–251.
[78] V. Hořejší, Anal. Biochem. 112 (1981) 1.
[79] A. Guttman, N. Cooke, Anal. Chem. 63 (1991) 2038.
[80] H.H. Heegaard, J. Chromatogr. A 680 (1994) 405.
[81] P. Sun, G.E. Baker, R.A. Hartwick, J. Chromatogr. 652 (1993) 247.
[82] H.D. Brede, E. Stevens (Ed.), Regulatory Control and Standardization of Allergenic Extracts, Gustav Fischer Verlag, Stuttgart, New York; 2nd Sept. 13th–15th, 1981; H.D. Brede, H. Goeing, M. Schaeffer (Ed.), 3rd International Paul-Ehrlich-Seminar, Sept. 8th–21st, 1983; M. Schaeffer, C. Sisk, H.D. Brede (Ed.), 4th October 16–17, 1985; R. Kurth (Ed.), 6th September 5–7, 1990; R. Kurth, D. Haustein (Ed.), 7th September 7–10, 1993.