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Determination of dissociation constant of phosphinate group in phosphinic pseudopeptides by capillary zone electrophoresis

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Abstract

Capillary zone electrophoresis (CZE) was used for determination of dissociation constant of phosphinate group in phosphinic pseudopeptides, i.e. peptides where one peptide bond is substituted by phosphinic acid moiety $-PO_2^- - CH_2-$. The dissociation constants were determined for a set of newly synthesized pseudopeptides derived from a structure N–Ac–Val–Ala$\psi(PO_2^- - CH_2)$Leu–His–NH_2 by nonlinear regression of experimentally measured pH dependence of their effective electrophoretic mobilities. CZE experiments were carried out in Tris–phosphate background electrolytes in the pH range 1.4–3.2. The pseudopeptides were synthesized as a mixture of four diastereomers, the separation of which was achieved in most cases. Moreover, differences of the effective mobilities of the pseudopeptide diastereomers enabled simultaneous determination of the dissociation constant of their phosphinate group without necessity of previous isolation of individual isomers.

Keywords: Dissociation constant; Phosphinic pseudopeptides; Phosphinate

1. Introduction

Phosphinic pseudopeptides represent a class of peptide isosteres where one peptide bond is substituted by enzymatically nonhydrolysable phosphinic acid moieties $-PO_2^- - CH_2-$ or $-PO_2^- -$ [1]. These compounds are intensively studied because of their promising biological activity—mimicking of the substrate transition state for at least two classes of proteolytic enzymes—Zn-metalloproteinases and aspartic acid proteinases [1,2].

Capillary zone electrophoresis (CZE) is currently widely used for high-performance separations of various peptides [3–5] including phosphopeptides, i.e. peptides containing phosphate esterified to the hydroxyl group of amino acids serine, tyrosine and threonine. Analyses of synthetic phosphopeptides [6,7] and of phosphopeptides originating from hydrolysis of phosphoproteins, e.g. casein [8,9] have been reported. However, to our knowledge CZE analyses of peptides with phosphorus within the peptide chain have not been yet reported. Two papers dealing with CZE separation of compounds partially similar to phosphinic pseudopeptides appeared when chiral analyses of various phosphonic and phosphinic acid analogues of phenylalanine and phenylglycine were performed [10,11].

CZE has emerged as a convenient and precise

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method for determination of acid dissociation constants (pK_a) of ionogenic groups of various types of compounds [12–23] including amino acids [24] and peptides [25,26]. The pK_a values were mostly determined in aqueous media, but recently the determination of pK_a in nonaqueous and mixed aqueous–organic solvents was reported [27–30]. CZE, unlike commonly used methods for pK_a determination such as potentiometric titration and ultraviolet spectroscopy, is capable of overcoming some drawbacks connected with these methods. CZE demands expressively lower amounts of analyte at lower concentration; therefore also pK_a of poorly soluble compounds can be determined. In the CZE method only migration time is measured, without the necessity of solute quantification or high purity degree of analyzed compound since impurities can be separated from the solutes of interest.

The aim of this study is to extend the applicability of CZE for determination of the dissociation constant of the phosphinic acid group in the set of pseudopeptide diastereomers derived from a structure N–Ac–Val–Alaψ(PO_2^-–CH_2)Leu–His–NH_2 indicated by its N- and C-terminal amino acid residues as VH. Derivatives with side chain modifications of VH are indicated as VH–A and VH–B, respectively, and derivatives with deprotected N- and/or C-terminus are indicated as N–VH, VH–O and N–VH–O, respectively (see Fig. 1).

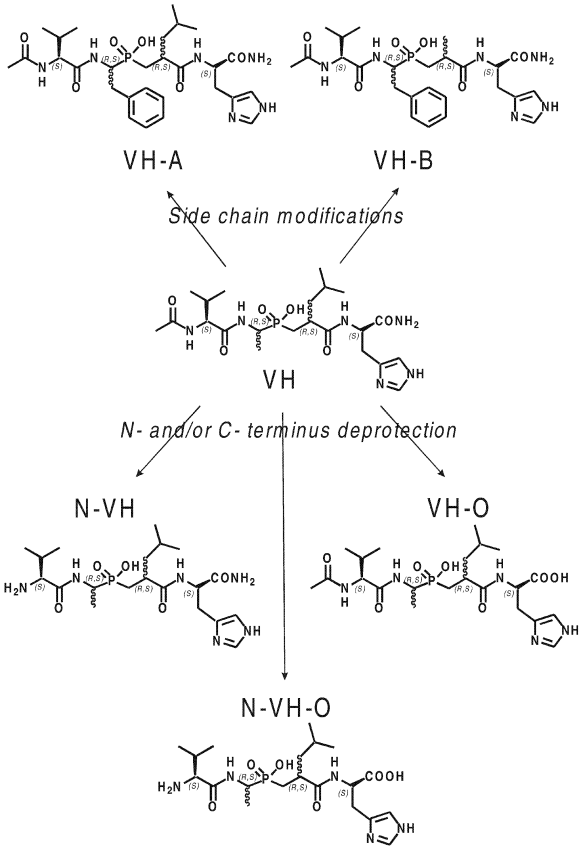


Fig. 1. Structures of analyzed phosphinic pseudopeptides (including stereochemical configurations) and their structural relationships.

2. Materials and methods

2.1. Reagents and chemicals

All chemicals were of analytical reagent grade. Phosphoric acid, potassium chloride and dimethylsulfoxide (DMSO) were obtained from Lachema (Brno, Czech Republic), Tris (tris(hydroxymethyl)aminomethane) was from Serva (Heidelberg, Germany). Phosphinic pseudopeptides were synthesized as a mixture of four diastereomers on a Rink Amide AM resin or 2-chlorotrityl resin (Calbiochem–Novabiochem, Läufelfingen, Switzerland) following the procedure of Yiotakis [31]. The protected phosphinic pseudopeptides precursors Fmoc–Alaψ[PO(OAd)–CH_2]Leu–OH, Fmoc–Pheψ-[PO(OAd)–CH_2]Leu–OH and Fmoc–Pheψ-[PO(OAd)–CH_2]Ala–OH were a kind gift of Dr Vincent Dive from Départment d'Ingénierie et d'Etudes des Protéines, CEA-Saclay, Gif sur Yvette, France. Prior to CZE analyses, the pseudopeptides were purified by HPLC (Waters, Milford, MA, USA) using a Vydac C_{18} 218TP510 column (Grace Vydac, Hesperia, CA, USA) and characterized by mass spectrometry and amino acid analysis.

2.2. CZE separation conditions

CZE experiments were carried out in a home made apparatus equipped with a UV detector monitoring absorbance at 206 nm. Data acquisition and handling were performed using the CSW Chromatography Station (DataApex, Prague, Czech Republic). Un-

treated fused-silica capillaries (I.D. 50 μm, O.D. 200 μm, total length 300 mm, effective length 190 mm) were supplied by the Institute of Glass and Ceramics Materials, Czech Academy of Sciences (Prague, Czech Republic). Separations were performed at ambient temperature (22–26 °C). Pseudopeptides were dissolved in deionised water in a concentration range of 0.7–2.5 m*M* and were introduced into the capillary hydrodynamically (pressure 700 Pa for 10 s).

In order to equilibrate the capillary, the following wash cycle was performed. The new capillary was flushed sequentially with water for 5 min, 0.1 *M* NaOH for 5 min, and water again for 2 min, followed by background electrolyte (BGE) for 2 min. Then, the BGE was left in the capillary overnight to equilibrate the inner fused-silica capillary surface and to stabilize the electroosmosis during the subsequent CZE runs. After rinsing the capillary with BGE for 0.5 min, it was conditioned by a 20-min application of the same high voltage as used in the respective runs. Between runs at the same pH, the capillary was rinsed with the BGE for 0.5 min. Before any change of the BGE, the capillary was rinsed with 0.1 *M* NaOH for 5 min and then stabilized using the above procedure.

BGE solutions were prepared by mixing the appropriate amounts of Tris and phosphoric acid in deionised water in the concentrations given in Table 1. BGEs were filtered through a 0.45-μm syringe filter (Millipore, Bedford, MA, USA) before use. Ionic strength of the BGEs was 25 m*M* except those composed of pure phosphoric acid. The higher ionic strength of phosphoric acid BGEs was unavoidable in order to reach pH values below 1.7.

2.3. Effective electrophoretic mobility determination

The effective electrophoretic mobility, m_{eff}, was determined from measurement of migration times of analyte, t_{mig}, and of neutral marker, t_{eof}. In all experiments DMSO was used as electroosmotic flow marker and m_{eff} was calculated from Eq. (1):

$$m_{\mathrm{eff}} = \frac{L_{\mathrm{t}} L_{\mathrm{d}}}{U}\left(\frac{1}{t_{\mathrm{mig}}} - \frac{1}{t_{\mathrm{eof}}}\right) \quad (1)$$

where L_{t} and L_{d} are total and effective capillary length, respectively; U is the applied separation voltage.

3. Theoretical

Dissociation constant of phosphinate group was determined in the pseudopeptides containing anionogenic phosphinic acid group and cationogenic histidine residue. The pseudopeptides without blocked C- and/or N-termini by a nonionic substituent have additional source of the charge in the carboxylic group (VH–O and N–VH–O) and in the amino group (N–VH and N–VH–O). The pseudopeptides with blocked N-terminus can be considered as monovalent bases whereas the pseudopeptides with unblocked N-terminus are in fact divalent bases.

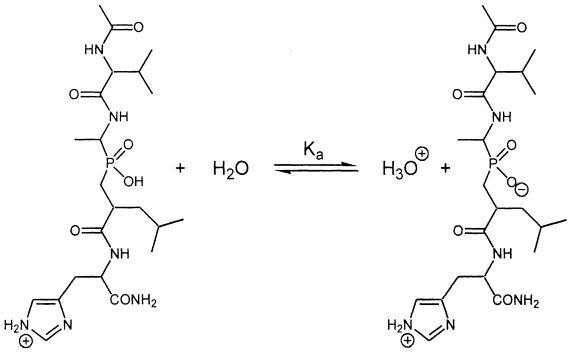
Table 1
pH, composition and ionic strength of the BGEs, separation voltage and input power used for phosphinate pK_{a} determination

pH	Phosphoric acid (m*M*)	Tris (m*M*)	Ionic strength (m*M*)	Voltage (kV)	Input power (W/m)
1.42	250	0	46	3.50	0.55
1.55	150	0	34	3.30	0.35
1.65	100	0	27	3.30	0.26
1.80	74	7	25	3.90	0.26
2.00	55	14	25	4.75	0.26
2.20	45	18	25	5.55	0.26
2.40	37	20	25	6.48	0.26
2.80	30	24	25	7.80	0.26
3.20	27	25	25	8.50	0.26

Owing to this difference a modified calculation procedure has to be employed for both groups of the pseudopeptides. Since the CZE analyses of pseudopeptides were performed at strong acid pH region, the dissociation of unprotected carboxylic group was not considered in the theoretical models of acid–base equilibrium.

3.1. Pseudopeptides with blocked N-terminus

The dissociation equilibrium of the phosphinic acid group in a pseudopeptide with protected N-terminus (VH taken as example) can be presented as follows:



(2)

The thermodynamic dissociation constant K_a can be expressed as:

$$K_a = \frac{a_{H_3O^+} a_{A^-BH^+}}{a_{HABH^+}} = \frac{a_{H_3O^+}\left[A^-BH^+\right]\gamma_{A^-BH^+}}{\left[HABH^+\right]\gamma_{HABH^+}} \quad (3)$$

where $a_{H_3O^+}$ is activity of hydroxonium ions, $a_{A^-BH^+}$ and a_{HABH^+} are activities of zwitterionic and cationic form of the pseudopeptide, respectively. Activities of species A^-BH^+ and $HABH^+$ can be expressed as a product of their concentrations $[A^-BH^+]$, $[HABH^+]$ and corresponding activity coefficients $\gamma_{A^-BH^+}$ and γ_{HABH^+}. The activity coefficient of zwitterionic and generally uncharged species A^-BH^+ is assumed to be equal to 1. The value of activity coefficient of charged species for aqueous solutions at 25 °C can be evaluated according to Debye–Hückel:

$$-\log\gamma = \frac{0.5085 z^2\sqrt{I}}{1 + 3.281 a\sqrt{I}} \quad (4)$$

where z is the charge and a is the effective hydrated diameter (nm) of the ion and I the ionic strength of the solution (mol/dm^3) defined as:

$$I = \frac{1}{2}\sum_i c_i z_i^2 \quad (5)$$

where c_i is molarity (mol/dm^3) and z_i is the charge of ith ion in the solution. As hydrated diameters of ions are seldom exactly known, the approximative value of $a = 0.5$ nm was used for calculations of activity coefficients in this paper.

It is favorable to define the apparent acidity constant K_a' as:

$$K_a' = \frac{a_{H_3O^+}\left[A^-BH^+\right]}{\left[HABH^+\right]} \quad (6)$$

The substitution of Eq. (6) in Eq. (3) with respect to unity value of $\gamma_{A^-BH^+}$ gives:

$$pK_a = pK_a' + \log\gamma_{HABH^+} \quad (7)$$

The effective electrophoretic mobility of analyzed pseudopeptides behaving like monovalent bases, m_{eff}, at low pH where the basic group is fully protonated can be expressed as:

$$m_{eff} = \alpha m_{HABH^+} + (1-\alpha)\, m_{A^-BH^+} \quad (8)$$

where $m_{HABH^+}$and $m_{A^-BH^+}$are the electrophoretic mobilities of respective species, α is the degree of dissociation defined as:

$$\alpha = \frac{\left[HABH^+\right]}{\left[HABH^+\right] + \left[A^-BH^+\right]} \quad (9)$$

As the electrophoretic mobility of the zwitterion A^-BH^+ is equal to 0, Eq. (8) and Eq. (9) can be combined to:

$$\frac{\left[A^-BH^+\right]}{\left[HABH^+\right]} = \frac{m_{HABH^+} - m_{eff}}{m_{eff}} \quad (10)$$

Substitution of this ratio in Eq. (6) by that in Eq. (10) gives:

$$m_{eff} = \frac{m_{HABH^+}}{1 + 10^{(pH - pK_a')}} \quad (11)$$

Eq. (11) is a particular case of the Boltzman sigmoidal decay function of the general form:

$$y = \frac{A_1 - A_2}{1 + e^{\frac{x - x_0}{dx}}} + A_2 \quad (12)$$

where x is the independent variable, y the dependent variable. Constants A_1 and A_2 are the initial ($y(-\infty)$) and final ($y(+\infty)$) values of the dependent variable, respectively; x_0 is center and dx is width of the sigmoidal curve.

When Eq. (12) is modified for dependence of effective electrophoretic mobility m_{eff} on pH, the following equation is obtained:

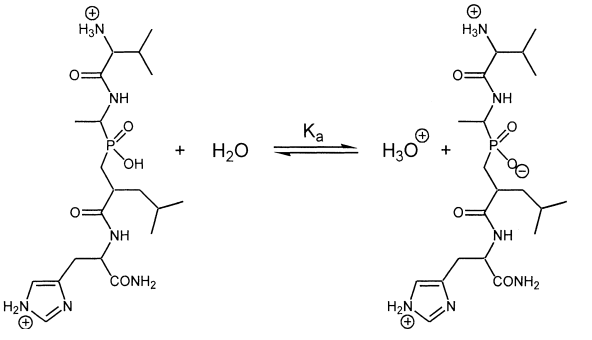
$$m_{eff} = \frac{m_1 - m_0}{1 + e^{\frac{pH - pK_a'}{dpH}}} + m_0 \quad (13)$$

where m_0 and m_1 are the electrophoretic mobilities of the deprotonated and protonated form of compound taking part in the dissociation equilibrium, respectively.

Eq. (13) represents a general mathematical model for determination of pK_a values by the fitting of experimental data.

3.2. Pseudopeptides with free N-terminus

The pseudopeptides with free amino group in the N-terminus which exist as doubly charged cations at highly acidic conditions become singly charged during deprotonation of the phosphinic acid group. The dissociation equilibrium of pseudopeptides N–VH is described as follows:



(14)

The thermodynamic dissociation constant K_a is then:

$$K_a = \frac{a_{H_3O^+} a_{A^-BH_2^{2+}}}{a_{HABH_2^{2+}}} = \frac{a_{H_3O^+}\left[A^-BH_2^{2+}\right]\gamma_{A^-BH_2^{2+}}}{\left[HABH_2^{2+}\right]\gamma_{HABH_2^{2+}}} \quad (15)$$

Relation analogous to Eq. (7) can be derived following the same procedure as that described in the above section:

$$pK_a = pK_a' + 3 \log \gamma_{A^-BH_2^{2+}} \quad (16)$$

The apparent acidity constant pK_a' of this type of pseudopeptides can be calculated utilizing the same model represented by Eq. (13) as used for the pseudopeptides behaving like monovalent bases.

4. Results and discussion

4.1. Selection of experimental conditions

The studied pseudopeptides carry positive net charge in the pH region close to the phosphinate pK_a due to positively charged residues of histidine and N-terminal amino group and due to mostly only partial dissociation of phosphinic acid group and almost negligible dissociation of carboxylic group. The CZE experiments leading to pK_a determination can be then carried out in the bare fused-silica capillaries in the cationic CZE mode. Because pK_a of phosphinate group is estimated to be 1.8 in related compounds [2], it is not possible to measure the mobility data in optimal pH interval with regard to reach very low pH region (pH$<$1) of fully protonated phosphinate group. CZE experiments in untreated fused-silica capillaries at strongly acid conditions are hampered by the large electric conductivity of the BGE leading to high Joule heat of applied electric field. Thus, lower voltage must be applied in order to keep input power and temperature inside the capillary at the same level as that of the low conductivity BGEs. The necessity of low voltage application along with small electroosmotic flow rate at low pH lengthen migration times, namely of EOF marker. Owing to these facts, 250 mM phosphoric acid (pH 1.42) was the most acid BGE used in this study.

BGEs of constant ionic strength (25 m*M*) were used in the pH interval 1.8–3.2. BGEs of pH below 1.8 exceeded the selected ionic strength; hence effective mobilities obtained in these BGEs were recalculated to 25 m*M* ionic strength according to a procedure given further in the text. Phosphoric acid and Tris were employed as buffer components because they produce in the given pH range univalent ions, which are consistent with Debye–Hückel theory utilized for calculation of activity coefficient and mobility correction for ionic strength.

In order to keep the temperature increase inside the capillary constant, experiments were carried out at constant input power of 0.26 W/m except for BGEs of pH 1.55 and 1.42 for which the higher input power was applied in order to avoid unacceptably long CZE experiments with low input power.

The upper pH value of BGE used for CZE of the pseudopeptides without free C-terminal carboxylic group was 3.2. At this pH the effective charge of the whole molecule of pseudopeptides became close to zero (peptides VH, VH–A and VH–B) or close to unity value (peptide N–VH) due to compensation of the positive charge from basic residues by the negative charge from almost fully dissociated phosphinate. Mobility data of the pseudopeptides with free carboxylic group were used up to pH 2.8. The effective charge of pseudopeptides VH–O became negative due to the dissociation of the carboxylic group causing impossibility to measure mobilities of VH–O above pH 2.8 by the used set-up. Mobilities of N–VH–O exhibited further decrease owing to the same reason above pH 2.8 and were not included into the calculation of phosphinate pK_a.

4.2. Correction of mobilities for dissipated power

All experiments were carried out in a home made device, which was not equipped with active cooling of the capillary. Therefore mobilities were determined at ambient temperature generally different from the standard temperature of 25 °C. This fact required the knowledge of the actual buffer temperature inside the capillary during the experiments and the employment of some correcting procedure for experimental mobilities. In order to compensate for the mobility shift due to the increased temperature inside the capillary, the following procedure, a modification of those reported in [32,33], was used. The electric current was measured in a wide range of voltages at the same set-up as that used for CZE experiments, only the capillary was filled with 0.02 *M* potassium chloride aqueous solution. The specific electric conductivity κ of the electrolyte solution was calculated from Eq. (17):

$$\kappa = \frac{L_t}{\pi R_i^2}\frac{i}{U} \tag{17}$$

where R_i is the inner radius of the capillary (m) and i is the electric current through the capillary (A).

At the lowest applied voltage, when thermal effects can be neglected, the conductivity of KCl solution at ambient temperature was obtained. When performing linear regression of tabulated conductivity data of standard a 0.02 *M* KCl solution in the temperature range from 15 to 35 °C [34], a linear relation is obtained with a regression coefficient $R = 0.99992$:

$$\kappa = 0.14304 + 5.36 \times 10^{-3}\, T \tag{18}$$

where κ is specific conductivity (S/m) and T is the temperature (°C).

The change of the electric conductivity at given power was recalculated to the temperature increment, ΔT, occurring at the corresponding power:

$$\Delta T = T_i - T_o = \frac{\kappa_{T_i} - \kappa_{T_o}}{5.36 \times 10^{-3}} \tag{19}$$

where T_i is average temperature inside the capillary (°C), T_o is ambient temperature during calibration (°C), κ_{T_i} is the conductivity of the electrolyte at given power applied and κ_{T_o} is the conductivity at the lowest power applied when the temperature increase inside the capillary is taken as negligible. A calibration plot relating the temperature increments of the solution inside the capillary to the applied power was constructed with the following estimation of the temperature increment: 4 °C at power 0.26 W/m; 6 °C at 0.35 W/m and 10 °C at 0.55 W/m. The measured effective mobilities were subsequently recalculated to a temperature of 25 °C assuming the mean increase of mobility to be 2.0% per Celsius

degree. This correction procedure is in more details described elsewhere [35].

4.3. Correction of mobilities for ionic strength

Due to the dependence of electrophoretic mobilities on ionic strength of BGE all experiments for determination of dissociation constants should be carried out in BGEs of constant ionic strength. BGEs of pH below 1.80 exceeded the selected value of 25 m*M* ionic strength; hence mobilities obtained in these BGEs and already corrected for temperature growth inside the capillary were recalculated to the values corresponding to the ionic strength of 25 m*M*. The approach describing deviations of mobilities from ideality was reported in Ref. [36]:

$$m = m^0 - \frac{\left(0.229m^0 + 3.12 \times 10^{-8}\right)\sqrt{I}}{1 + 3.28a\sqrt{I}} \qquad (20)$$

where m^0 ($m^2/V/s$) is the limiting mobility, I the ionic strength (mol/dm^3) and a is the diameter of the counterion (nm). Eq. (20) was experimentally verified to be valid for uni-univalent aqueous solutions of electrolytes over an ionic strength interval up to 75 m*M* [36]. Thus, Eq. (20) allows recalculation of the mobility values obtained in the BGEs of different ionic strength to the same ionic strength within the validity range.

4.4. Electrophoretic behavior of phosphinic pseudopeptides

Dependencies of effective mobilities of diastereomers of analyzed pseudopeptides were obtained in the pH interval 1.4–3.2. As can be seen in Fig. 2, lower pH led to bigger mobility difference and better resolution among diastereomers of majority of the pseudopeptides, only in the case of the pseudopeptide N–VH–O this tendency was opposite (see Fig. 2d). Insight into the effective mobilities brings the following conclusions:

- The close values of effective mobilities of VH–O and VH indicate that free carboxylic group of VH–O remains almost fully protonated below pH 2 (see Fig. 2c and a).
- The pseudopeptides with free N-terminus (N–VH and N–VH–O) exhibit distinctly higher mobilities comparing to VH due to higher effective charge (see Fig. 2b, d and a).
- Effective mobilities of the pseudopeptides VH, VH–A and VH–B decrease with increasing size of the molecules, i.e. in order VH > VH–B > VH–A (see Fig. 2a, f and e).

4.5. Dissociation constants calculation

The calculation of apparent dissociation constant pK_a' values were accomplished by non-linear fitting of experimental data to Eq. (13) using the computer program Origin 6.1 (OriginLab, Northampton, MA, USA) because this approach was verified to give the least biased determination [21] in comparison with other approaches such as linear regression of linearised exponential model, which describes relationship between mobilities and pH [12,14], or point-to-point calculation utilizing the Henderson–Haselbalch equation [21]. Thermodynamic dissociation constants, pK_a, of separated diastereomers were consecutively obtained from apparent dissociation constants pK_a' using either Eq. (7) for the pseudopeptides with blocked N-terminal amino group or Eq. (16) for the pseudopeptides with free N-terminus. The obtained pK_a values are presented in Table 2. Dissociation constants of comigrating diastereomers of pseudopeptides VH–1 and VH–2, VH–3 and VH–4, VH–A–2 and VH–A–3 were assumed to be equal. The determined pK_a values of phosphinate group in the analyzed phosphinic pseudopeptides are generally in a good agreement with the published value of 1.8 for related phosphinates [2].

5. Conclusions

This work outlines suitability of CZE for determination of dissociation constants of phosphinate group in the phosphinic pseudopeptides. Additionally, due to the achieved separation of diastereomers, simultaneous determination of their dissociation constants was possible without their previous preparative separation, which demonstrates the usefulness of CZE as a separation technique for obtaining im-

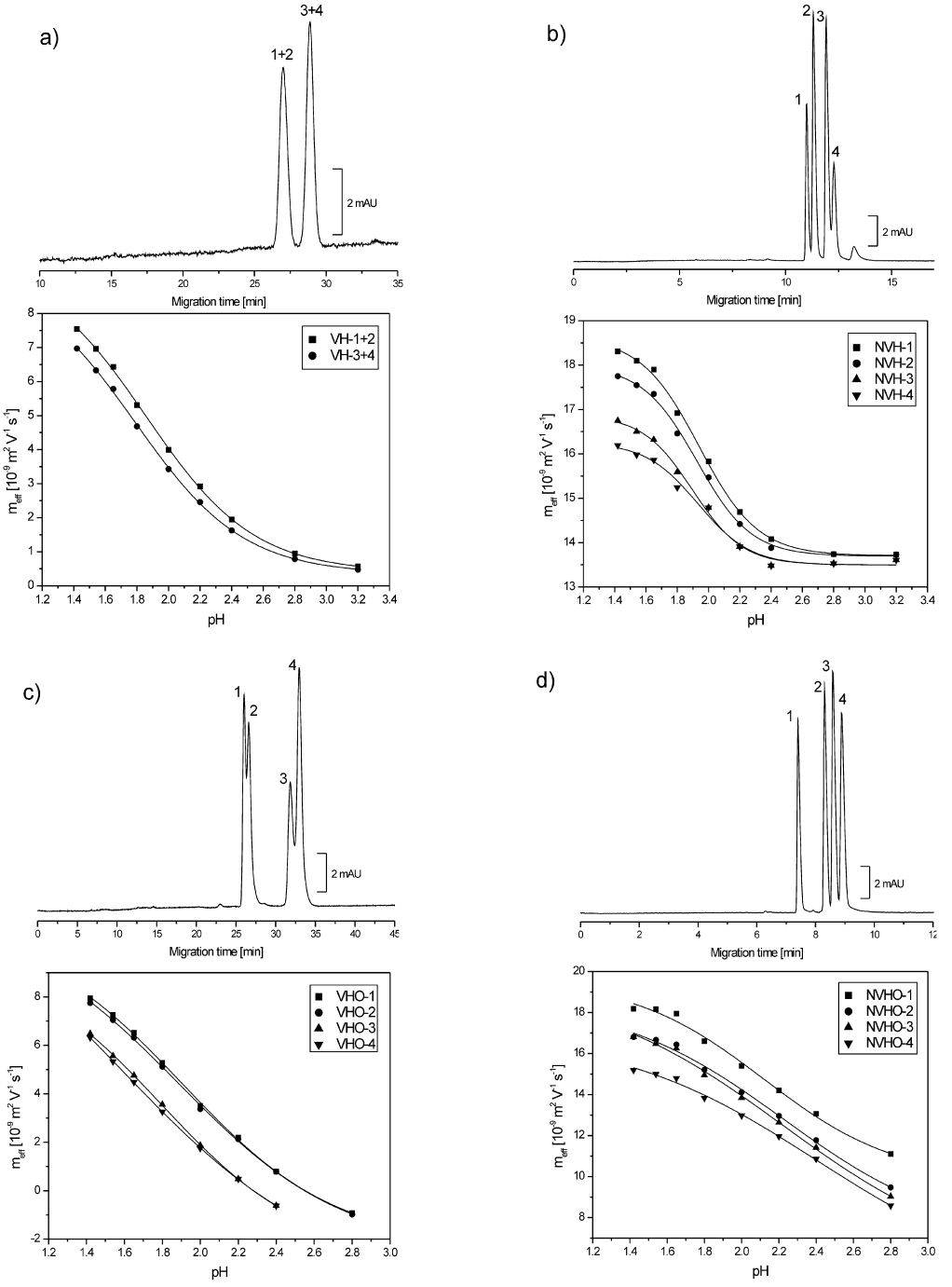


Fig. 2. Dependencies of effective mobilities, m_{eff}, of separated pseudopeptide diastereomers on pH with example of electropherogram at the best achieved resolution of diastereomers in Tris–phosphate BGEs at indicated pH. The pseudopeptides are indicated by abbreviations given in Fig. 1; peaks of the individual diastereomers are numbered in the order of decreasing electrophoretic mobility: (a) VH, electropherogram at pH 2.40, (b) N–VH, electropherogram at pH 1.42, (c) VH–O, electropherogram at pH 1.55, (d) N–VH–O, electropherogram at pH 2.80, (e) VH–A, electropherogram at pH 2.00, (f) VH–B, electropherogram at pH 1.55. Capillary 50 μm I.D./200 μm O.D., total/effective length 300/190 mm, UV detection at 206 nm, injection 700 Pa for 10 s, sample concentration 0.7–2.5 m*M*, voltage—see Table 1.

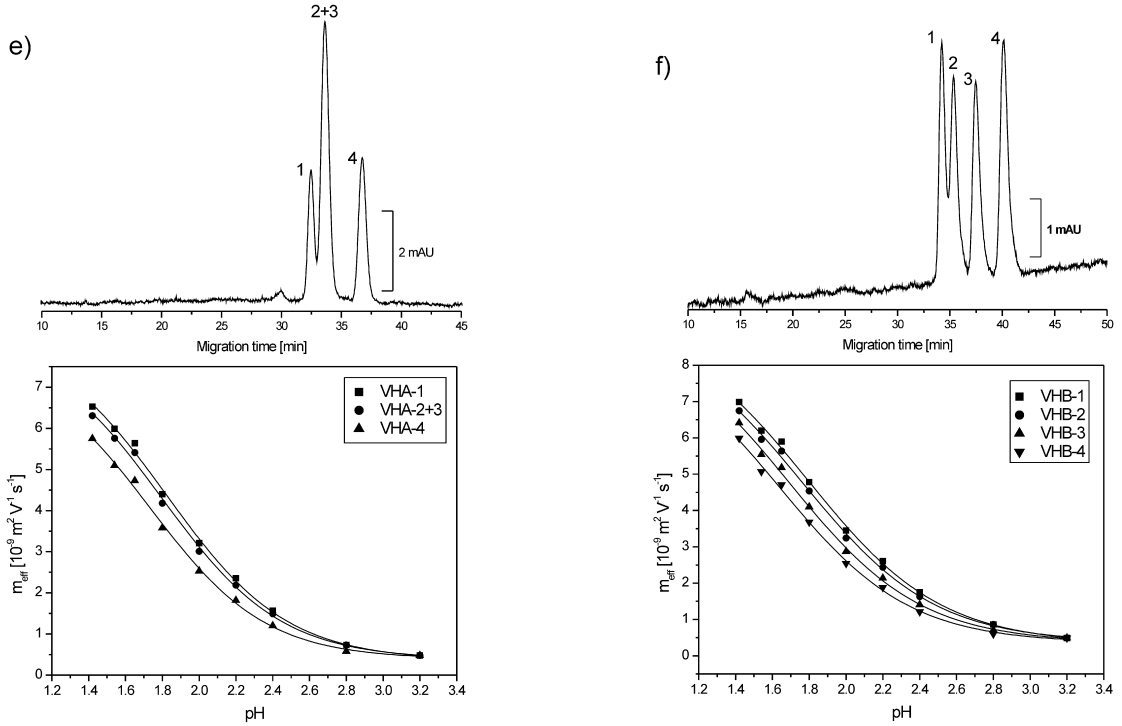


Fig. 2. (*continued*)

Table 2
Determined values of pK_a of phosphinate group in separated diastereomers of the pseudopeptides

Pseudopeptide	pK_a (1)	pK_a (2)	pK_a (3)	pK_a (4)
VH	1.80±0.03	1.80±0.03	1.73±0.04	1.73±0.04
N–VH	1.75±0.02	1.73±0.02	1.71±0.03	1.74±0.03
VH–O	1.84±0.03	1.82±0.04	1.73±0.04	1.52±0.20
N–VH–O	1.93±0.05	2.03±0.08	2.03±0.10	2.21±0.23
VH–A	1.76±0.05	1.73±0.06	1.73±0.06	1.67±0.07
VH–B	1.73±0.07	1.74±0.08	1.63±0.09	1.58±0.11

portant physicochemical characteristics of biomolecules.

Acknowledgements

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