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Characterization of jeffamines by capillary isotachophoresis and zone electrophoresis

J.L. Beckers*, Th.P.E.M. Verheggen, H.J.P. Triepels, F.M. Everaerts

Eindhoven University of Technology, Laboratory of Instrumental Analysis, P.O. Box 513, 5600 MB Eindhoven, The Netherlands

Abstract

Isotachophoresis has the unique feature that both qualitative and quantitative information can be deduced from step heights in isotachopherograms, even for unknown ionic species. For strong cations and anions there exists a linear relationship between, respectively, the measured step heights and the response factor (RF) versus the specific zone resistance at 25°C (SZR_{25}) for a specific leading electrolyte. Applying two standard components, e.g., the leading ion and Na^+, the relationship between measured step heights and the SZR_{25} values can be set up and from the step heights of the sample ionic species in the isotachopherograms the SZR_{25} values can be obtained. With these values all other parameters such as the mobilities, the concentrations in the zones and the RF values can be calculated. In combination with the measured zone lengths, the molar fractions and mass fractions can be calculated. In this way, the number-average and weight-average molecular masses can be obtained for homologous series of anions/cations from the isotachopherograms of the mixtures. This procedure is applied to jeffamines D230 and D400 and the obtained molecular masses are comparable with the labelled values.

Keywords: Isotachophoresis; Molecular mass determination; Jeffamines; Amines

1. Introduction

Most artificial tissue valves are porcine aortic heart valves, consisting for the larger part of collagen and elastin. These components provide the valves with the needed mechanical strength and flexibility. Other components of the valves are glycosaminoglycans, proteoglycans lipids, cells, enzymes and proteins. Because the time between removal and usage of the porcine valves is several days, cellular units, proteoglycans, enzymes and proteins will be degraded and crosslinking is necessary to give the collagen-based tissue the desired mechanical strength and to prevent fast degradation. For such purposes mixtures of cationic and/or anionic species are often used.

For the crosslinking of collagen-based material mixtures of bifunctional jeffamine-spacer (jeffamine D230) can be used [1]. See Fig. 1 for the structural formula of jeffamines. The reaction involves the activation of the free carboxylic acid groups of collagen by 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide·HCl (EDC) and *N*-hydroxysuccinimide (NHS) followed by reaction of these activated carboxylic acid groups with the amine groups of the bifunctional jeffamine-spacer to form the crosslinks. To determine the amount and the sort of diamines used for the crosslinking, the crosslinked collagen samples can be hydrolyzed, after which the liberated diamines have to be analyzed. Therefore, it is very

*Corresponding author. Tel.: +31-40-4-73021, Fax: +31-40-4-53762.

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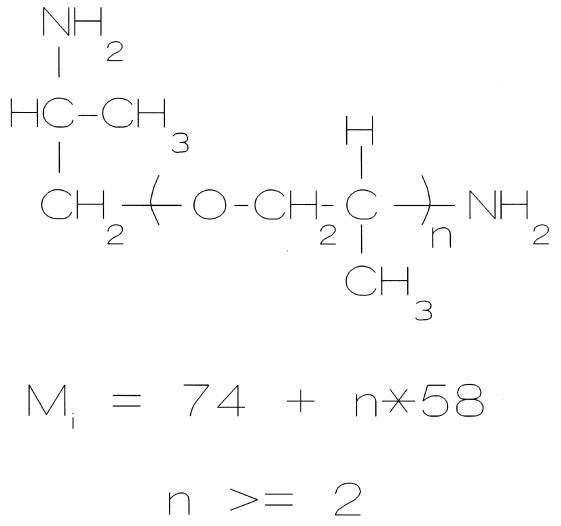

Fig. 1. Structural formula for jeffamines.

important to characterize the mixture of diamines before and after the crosslinking.

Mixtures of anions/cations can be characterized by their number-average (M_n) or weight-average (M_w) molecular mass [2]. These molecular masses can be obtained applying size-exclusion chromatography (SEC) [3]. When applying SEC, a problem is always the determination of the relationship between molecular mass vs. retention time. In this article isotachophoresis (ITP) is used for the determination of M_n and M_w of jeffamines.

2. Theory

The separation mechanism in ITP is well defined [4,5] and by applying a mathematical model all parameters in sample zones can be calculated if the p*K* values and the mobilities of sample ionic species are known. For not too high applied electric current densities a linear relationship between step heights and the specific zone resistance at 25°C (SZR_{25}) is obtained applying a.c. conductivity detection [6]. If for two standard components, e.g., the leading ion and sodium ions, with known p*K* values and mobilities, the SZR_{25} values are calculated with a mathematical model, the linear relationship between the measured step heights and the calculated SZR_{25} values can be set up. With this relationship the SZR_{25} value of an unknown ionic species can be obtained from its step height in the isotachopherograms and for strong anions/cations the mobility of the component can be obtained from a single experiment. As an example in Fig. 2, the relationship between measured step heights, obtained from Ref. [6], and calculated SZR_{25} values are given for a leading electrolyte consisting of 0.01 *M* potassium acetate at pH 5. Applied electric current was very low (2 μA) in order to avoid an extensive Joule heating. See Table 1 for used p*K* values and mobilities in the calculations.

Also, the concentration in the sample zones or the response factor (RF) can be determined. The RF (C/mol) [7,8] represents the slope of a calibration graph of the product of the zone length ZL (s) and the applied current *I* (A) versus the amount of the sample component *Q* (mol):

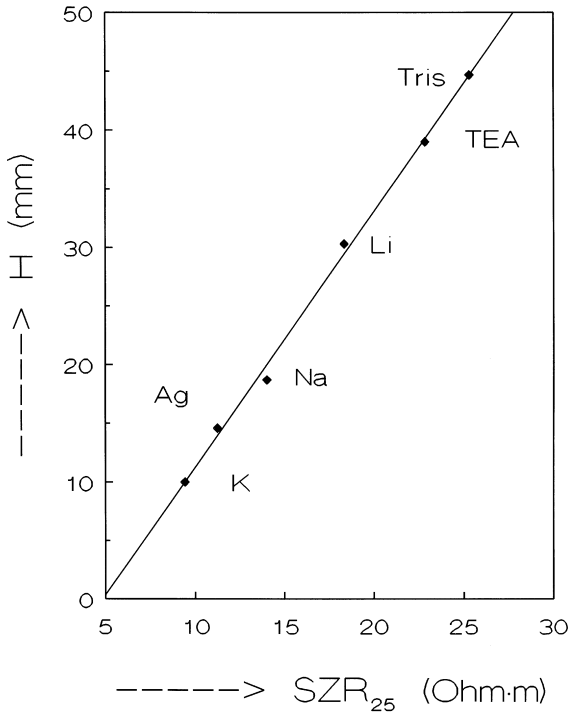

Fig. 2. Relationship between measured step heights *H* and calculated specific zone resistance SZR_{25} values for some cations applying a leading electrolyte 0.01 *M* potassium acetate at pH 5. Measured values are obtained from Ref. [6].

Table 1
Mobilities at infinite dilution m and pK values for ionic species, used in the calculations

Ionic species	$m \cdot 10^9$ (m^2/V s)	pK
Acetic acid	−42.4	4.76
Lithium	40.1	–
Potassium	76.2	–
Silver	64.2	–
Sodium	51.9	–
TEA[a]	32.5	–
Tris[b]	29.5	8.10

[a] TEA = Tetraethylammonium.
[b] Tris = Tris(hydroxymethyl)aminomethane.

$$RF = \frac{ZL \cdot I}{Q} \quad (1)$$

The concentrations in the sample zones in ITP are not constant. The concentrations in the zones decrease for decreasing mobilities (higher SZR values). This means that for equimolar mixtures of cations the zone lengths, and by this also the RF values, increase for lower mobilities. In Fig. 3 the relationship between the RF values (calculated from the measured zone lengths) and calculated SZR_{25} values is given for the same cations and electrolyte system as used in Fig. 2 injecting an equimolar sample mixture. The concentration of the sample ions was 0.00154 M and the injected volume was 1 μl. In Table 2 all measured step heights, measured zone lengths and calculated SZR_{25} and RF values are given for the cationic species of Figs. 2 and 3. The measured zone length for the Na^+ zone is too long, because of the presence of Na^+ ions in the applied electrolytes. After correction for the Na^+ in the blank, the RF value fits the relationship of Fig. 3.

2.1. How to determine the M_n and M_w of a mixture of ionic components?

For a mixture of strong cations/anions, i.e., known pK values, the M_n and M_w can be determined in a single run in the following way. An isotachopherogram is measured by injecting the sample solution. Injection volume is not important. If the mixture contains a standard ionic species, e.g., Na^+, we can use the step heights of the leading ion and that of the

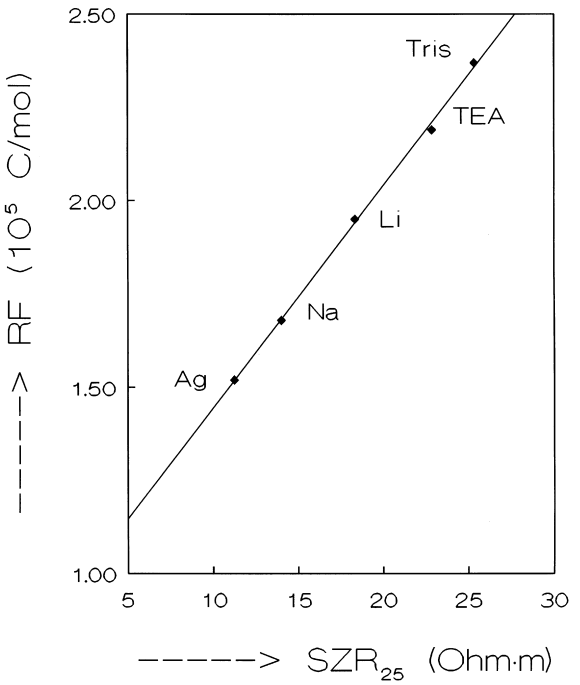

Fig. 3. Relationship between response factors RFs, calculated from measured zone lengths ZLs, and calculated SZR_{25} values for the same cations and leading electrolyte as used in Fig. 2, for an equimolar sample mixture.

standard to set up the relationship between step heights and SZR_{25} values. For each component, the SZR_{25} can be calculated from its step height using this relationship. From the SZR_{25} value the mobility of the ionic species and the concentration in its zone

Table 2
Measured step heights H, measured zone lengths ZLs and from the measured step heights and zone lengths calculated specific zone resistance SZR_{25} and response factor, RF, values for some cationic species, applying a leading electrolyte, LE, of 0.01 M potassium acetate at pH 5

	H (mm)	SZR_{25} (Ω·m)	ZL (mm)	RF (10^5 C/mol)
K^+	10.0	9.42	–	–
Ag^+	14.6	11.24	117	1.52
Na^+	18.7	14.01	199	1.68
Li^+	30.3	18.34	150	1.95
TEA^+	39.0	22.86	169	2.19
$Tris^+$	44.7	25.33	183	2.37

can also be calculated. For all zones the amount Q (mol) of the ionic species present is:

$$Q = Ac_i l_i \tag{2}$$

whereby A is the capillary cross-section, c_i is the calculated sample concentration in the zone and l_i is the length of the zone, measured from the isotachopherogram.

The molar fraction x_i can be calculated as:

$$x_i = \frac{c_i l_i}{\sum_i c_i l_i} \tag{3}$$

If the molecular mass M_i is known for all fractions, M_n can be calculated as:

$$M_n = \sum_i x_i M_i \tag{4}$$

Also M_w can be calculated using:

$$w_i = \frac{x_i M_i}{\sum_i x_i M_i} \text{ and } M_w = \sum_i w_i M_i \tag{5}$$

whereby w_i is the mass fraction, that can be calculated from the molar fractions.

3. Experimental

3.1. Isotachophoresis

For all ITP experiments, laboratory-made equipment was used as described and built by Everaerts et al. [4]. The separation compartment consisted of a PTFE capillary tube (Habia, Breda, Netherlands) with an I.D. of 0.4 mm and a separation length of 35 cm. Distance between injection and detection was 28 cm. The zones were detected with a laboratory-made high-performance a.c. conductivity detector. The power supply was of type HCN 35-500 (F.U.G., Electronic, Rosenheim, Germany). Injection was made with a syringe. For all experiments the constant electric current was 70 μA and the injection volume was 0.8 μl, unless stated otherwise.

3.2. Zone electrophoresis

For all capillary zone electrophoresis (CZE) experiments an apparatus was used consisting of a Prince injector (Prince Technologies, Emmen, Netherlands) combined with a power supply (type HCN 35-500, F.U.G., Electronic) and a Spectra 100 variable-wavelength UV absorbance detector (SpectraPhysics, San Jose, CA, USA). The separation capillary tube was made of uncoated fused-silica (Siemens, Mühlheim, Germany, I.D. 75 μm) with total length of 57.8 cm and a distance between injection and detection of 51.0 cm. The wavelength of the UV detector was set at 220 nm. All zone electrophoretic experiments were carried out in the cationic mode applying a constant voltage of 20 kV. The injection pressure was 15 mbar and the injection time 0.15 min. The outlet side of the capillary tube was connected with an electrode compartment described in Ref. [9], through which the apparatus could be applied in both the open and closed mode. For the separations the system was used in the open mode.

3.3. Data acquisition

For the data acquisition of both the isotachophoretic and zone electrophoretic experiments the software used was the Caesar program (B*wise, Geleen, Netherlands) running on an Olivetti M250 computer in combination with the multilab interface (TUE-TS, Eindhoven Technical University, Eindhoven, Netherlands).

3.4. Solutions

For the zone electrophoretic experiments a background electrolyte (BGE) was used consisting of a mixture of 0.01 *M* histidine and 0.01 *M* 2-(*N*-morpholino)ethanesulfonic acid (MES) at pH 6 (indirect UV mode).

For the isotachophoretic experiments a leading electrolyte was used consisting of 0.01 *M* KOH and

acetic acid at pH 4.1. The terminator solution was 0.01 *M* acetic acid (hydrogen ions as terminating ions).

3.5. Chemicals

Chemicals were all of analytical-reagent grade and purchased from Merck (Darmstadt, Germany). For the preparation of all solutions ultra-pure water was used.

4. Results and discussion

To check the foregoing theory, isotachopherograms are measured for jeffamines D230 and D400 applying a leading electrolyte of potassium acetate at pH 4.1 and a terminating solution of 0.01 *M* acetic acid. The applied electric current was 70 μA and the injection volume by syringe was 0.8 μl. The used solution of jeffamines contained some Ca^{2+} and Na^{+}. In Fig. 4 the isotachopherograms of jeffamines (a) D400 and (b) D230 are shown. For the calculations the baseline of the leading ion K^{+} and the step height of Na^{+} are taken as the two standards. The first step height (1) must belong to the jeffamine fraction with molecular mass 190 which is a large part of the D230 sample and a minor part for the D400. In total, eight jeffamine fractions could be distinguished in D400 with ITP. A ninth zone is just visible in the isotachopherogram. The first and ninth zones are not quantified in the calculations. The step heights of the jeffamine fractions in both isotachopherograms are reproducible. From the step heights in the isotachopherograms the mobilities at infinite dilution and the concentrations in the zones are calculated and all values are given in Table 3. From the measured zone lengths l_i and calculated concentrations in the zone c_i for all fractions, the molar fractions x_i and mass fractions w_i are calculated and with Eqs. (4) and (5), the values for M_n and M_w are obtained. The values of M_n and M_w are easily obtained and are comparable with the labelled values of 230 and 400 given by the manufacturer.

We also tried to separate the jeffamines mixture

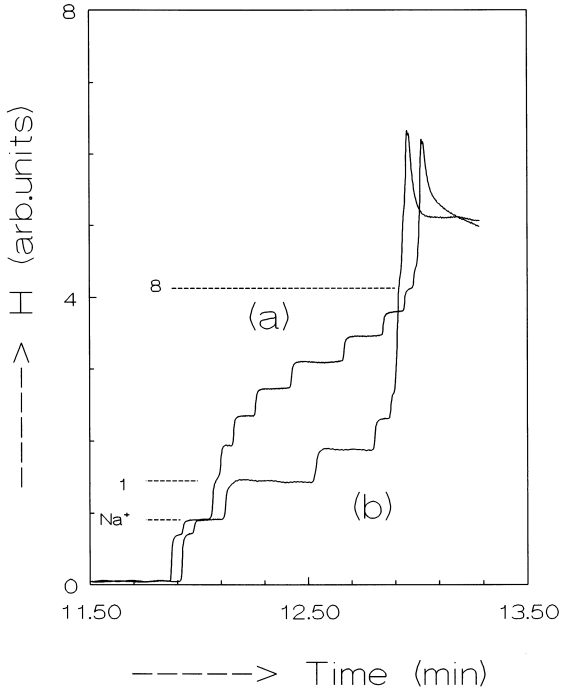

Fig. 4. Isotachopherograms for the separation of the mixtures of the jeffamines (a) D400 and (b) D230. In the calculations the two standards are the leading ion K^{+} and Na^{+}. The numbers 1 and 8 refer to the value of $n-1$ of the jeffamine composition (see Fig. 1). Leading electrolyte was 0.01 *M* potassium acetate at pH 4.1 and the terminator was 0.01 *M* HAc. For further information see Section 3.

with CZE. Because of lack of UV absorbance properties of the diamines, we used as BGE a solution of 0.01 *M* histidine and 0.01 *M* MES at pH 6. A good separation in the indirect UV mode could easily be obtained. In Fig. 5 the electropherogram for the separation of D400 in CZE is given. Besides the nine peaks of the jeffamines between ca. 3.5 and 4.8 min some other peaks are present in the electropherogram not visible in the isotachopherogram and remarkable is a second series of peaks between ca. 5.2 and 6 min, with lower mobilities i.e., jeffamines with higher *n* values. From the peaks present in the electropherogram it is also possible to calculate the M_n and M_w although an exact calculation can be troublesome because the transfer ratio is not a constant for all fractions [10]. For all peaks

Table 3
Molecular mass M, mobility at infinite dilution m, concentration in the sample zones c, measured zone length l_i and molar fraction x_i for the components of the jeffamines mixtures D230 and D400 in the ITP sample zones 1–8 of the measured isotachopherograms

Peak No.	M (g/mol)	$m \cdot 10^9$ (m^2/V s)	c (mol/l)	D230		D400	
				l_i (mm)	x_i	l_i (mm)	x_i
1	190	48.3	0.00382	24.4	0.57		
2	248	42.8	0.00350	16.1	0.34	3.55	0.08
3	306	39.4	0.00318	4.4	0.09	6.10	0.13
4	364	35.4	0.00295			9.85	0.20
5	422	32.9	0.00278			14.5	0.27
6	480	30.9	0.00255			10.80	0.19
7	538	29.3	0.00241			5.85	0.10
8	596	27.9	0.00224			2.6	0.04
			M_n =	219.9		409.7	
			M_w =	226.3		428.9	

such a transfer ratio can be calculated with a mathematical model if the mobility of the electro-osmotic flow m_{eof} is known.

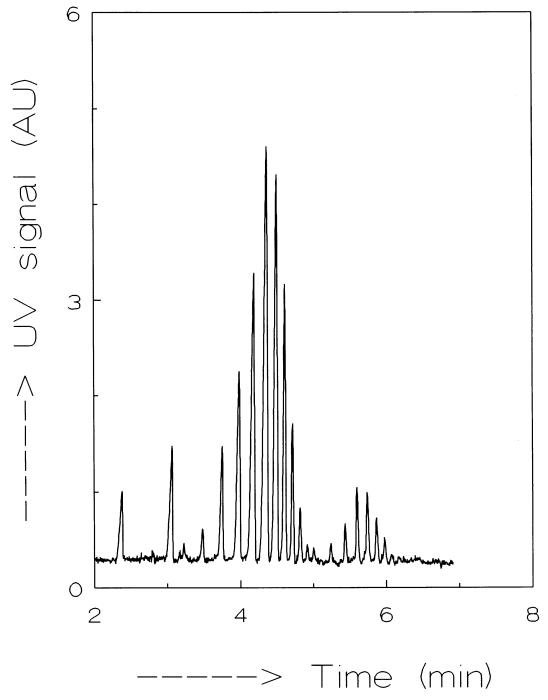

Fig. 5. Zone electropherogram for the separation of jeffamine D400, applying a BGE consisting of 0.01 M histidine–MES at pH 6. For further information see Section 3.

5. Conclusions

Separation of mixtures of homologous series of anions/cations is possible with both ITP and CZE. For ITP experiments both quantitative and qualitative information can be obtained from step heights and for strong anions/cations (i.e., if the pK values of the sample ions are known) from a single experiment all zone parameters can be calculated applying two standards. From the zone concentration and the zone lengths in the isotachopherogram mixtures of homologous series of anion/cations can be characterized by calculation of their M_n and M_w values. Calculations for D230 and D400 show good results. In this method the concentration in the sample zones and transfer ratio are directly deduced from the step height, also for unknown components. In principle an analogue procedure can be followed for CZE experiments. Then, however, the m_{eof} should be known and the transfer ratio must be treated with a mathematical model. The last procedure is currently under investigation.

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