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QUANTITATION IN ISOTACHOPHORESIS

THE CONCEPT OF RELATIVE CORRECTION FACTORS

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SUMMARY

Based on the moving boundary equation for weak electrolytes, a relationship is derived between the amount of an ionic species in an isotachophoretic zone and the corresponding zone length in the isotachopherogram, and the concept is introduced and investigated of the relative correction factors, which permits the quantitative interpretation of isotachopherograms obtained under variable working conditions. The relative correction factor, $D_{X,R}$, has been proved to be constant to within 1 % (relative) for a series of organic acid anions for four leading electrolytes that differ widely in their net mobilities and concentrations.

INTRODUCTION

The problems involved in quantitative analysis by isotachophoresis have been given relatively little attention[1–3] and published papers have been concerned with the statement of direct proportionality between the amount of an ionic species and the corresponding zone length. In such a case, it is essential for each proportionality constant to be determined by a separate calibration. The problem of calibration has been reduced to determination of a universal calibration constant[3] representing the free cross-section of a column and the amount of any given ionic species is determined as the product of the calculated theoretical adjusted concentration of the species in its zone, the respective zone length and the universal calibration constant. The principle of both methods is simple, but their applicability is limited to instances in which identical and strictly constant working conditions can be ensured during both the calibration and the analysis, in particular the qualitative and quantitative composition of the leading electrolyte, the density of the driving electric current, the hydrodynamic counterflow and the temperature. Further, if the universal calibration constant[3] is employed, one must know the absolute values of the concentrations of all ionic species present in the leading electrolyte and all data necessary for calculating adjusted concentrations, such as ionic mobilities and dissociation constants. In addition, it is essential to know the absolute amount of sample used.

It is often difficult to satisfy all these requirements, for example, in the analysis of

samples that contain species the effective mobility of which is higher than that of the leading ionic species under given working conditions. In such a case, during the separation process, the faster species enters the leading electrolyte zone and changes the leading zone composition. The concentrations of the sample components in their respective zones adjust themselves to a new mixed leading electrolyte and their actual values can differ substantially from the values theoretically predicted or determined by calibration with a standard mixture. In addition, changes in leading electrolyte composition can also be caused by some artifacts entering the leading zone from the electrode chambers through membranes and/or changes can arise directly in the leading zone owing to the selective permeability of membranes (*cf.*, ref. 4). Another problem is the presence of impurities in the leading electrolyte, when deviations of the actual composition from the theoretical composition occur as systematic errors in the calculated adjusted concentrations. Moreover, both of the previously mentioned methods of quantitative analysis, based on the use of absolute calibration data, may suffer from large errors if the calibration data measured under given working conditions are used for interpreting isotachopherograms obtained at different conditions, *e.g.*, at a different electric driving current. This last limitation pertains even to the use of the universal calibration constant (*cf.*, ref. 3).

It is therefore essential to carry out quantitative isotachophoresis by other techniques that eliminate some of the above problems. In particular, the calibration data should be of more general use, *i.e.*, calibration data measured under a particular set of working conditions should be valid under a different set of working conditions. These requirements are realized by utilizing the concept of the relative correction factors and by techniques of quantitative analysis based upon this concept as it is commonly used in quantitative analysis by gas chromatography[5].

THEORETICAL

Concept of relative correction factors

A basic reason for using correction factors is to simplify relationships between the amount of an ionic species in its zone in the column and the corresponding length in the isotachopherogram in such a way that we can convert any zone length recorded in the isotachopherogram into arbitrary mole units by using a simple factor dependent only upon the respective ionic species. It is therefore necessary to define in a quantitative way a suitable parameter —correction factor— the magnitude of which is characteristic of the given species. A zone β of an ionic species X is represented by the solution of this species, the concentration of which in it is $c_{X,\beta}$. This solution occupies a certain volume, $V_{X,\beta}$, in the column. Provided that the composition of the leading electrolyte (zone α) is given, the concentrations of all species under separation in their respective zones are fixed, irrespective of both the magnitude of the electric driving current and the hydrodynamic counterflow (*cf.* ref. 6). This means, for a given leading electrolyte, the volume $V_{X,\beta}$ represents uniquely the moles of species X, n_X, present in zone β, and it is given by the expression

$$V_{X,\beta} = \frac{1}{c_{X,\beta}} \cdot n_X \qquad (1)$$

However, the quantity $c_{X,\beta}$ depends on the composition of the leading electrolyte. The

following considerations are intended to eliminate this specific quantity $c_{X,\beta}$ by substituting it with appropriate quantities the values of which are either directly available from experiments or are constant throughout the whole separation process.

The moving boundary equation for weak electrolyte systems[7,8] can be written (see ref. 9) as:

$$\frac{u_{X,\alpha} \cdot c_{X,\alpha}}{\kappa_\alpha} - \frac{u_{X,\beta} \cdot c_{X,\beta}}{\kappa_\beta} = v_{\alpha,\beta} (c_{X,\alpha} - c_{X,\beta}) \tag{2}$$

The quantity $v_{\alpha,\beta}$ is the volume in millilitres through which the boundary moves per coulomb passed. The specific conductances of the two zones are κ_α and κ_β. The quantities $c_{X,\alpha}$ and $c_{X,\beta}$ are concentrations (moles $\cdot$ l^{-1}) and $u_{X,\alpha}$ and $u_{X,\beta}$ are net mobilities[10] of the ionic species X in zones α and β, respectively. Isotachophoresis is a special case of the moving boundary system, where, with the exception of buffering counterionic species, each separated ionic species is present in one zone only and, at the same time, all zones migrate with the same velocity. If the buffering counterionic species is denoted by the symbol B and the leading ionic species and leading zone are designated as A and α, respectively, and, further, the symbols X, R, S, ... and β, ϱ, σ, ... represent ionic species under separation and their zones, respectively, then it is possible to describe the isotachophoretic migration of this system in the following way:

$$v_{\alpha,\beta} = v_{\beta,\varrho} = v_{\varrho,\sigma} = \ldots\ldots = \frac{u_{A,\alpha}}{\kappa_\alpha} = \frac{u_{X,\beta}}{\kappa_\beta} = \frac{u_{R,\varrho}}{\kappa_\varrho} = \ldots \tag{3}$$

Suppose that the zone β containing species X moves along the column while the electric current is I (C/sec) and the hydrodynamic counterflow is H (ml/sec). Then the zone β proceeds along the column at a rate, expressed in millilitres per second, equal to $I \cdot v_{\alpha,\beta} - H$ and the time, t_X, required for passing the whole volume of zone β, $V_{X,\beta}$ (ml), through the detector is given by

$$t_X = \frac{V_{X,\beta}}{I \cdot v_{\alpha,\beta} - H} \tag{4}$$

In the isotachopherogram, the length, L_X, corresponding to the zone is $b \cdot t_X$, where b is the chart speed. Relating the zone volume, $V_{X,\beta}$, to 1 mole of ionic species X and denoting it by d_X, then $d_X = V_{X,\beta}/n_X$ and, by using eqn. 4, we can express the zone length in an isotachopherogram corresponding to 1 mole of species X in the following way:

$$\frac{L_X}{n_X} = \frac{b}{I \cdot v_{\alpha,\beta} - H} \cdot d_X \tag{5}$$

The first term of the right-hand side of eqn. 5 represents a non-specific constant, the value of which is independent of the ionic species under analysis. On the other hand, the quantity d_X (which can be called the "molar dilution of species X", representing the volume of a zone in which 1 mole of the species X is present) is a specific constant for the species X under given working conditions.

If an analysis is carried out under constant working conditions, the non-specific constant is relevant to all ionic species separated in the same way and it is therefore

convenient to eliminate it. It can be eliminated by introducing a quantity called the "relative molar dilution",[1] $D_{X,R}$, defined as the molar dilution of the species X relative to that of a suitable reference species R separated simultaneously. Thus,

$$D_{X,R} = \frac{d_X}{d_R} = \frac{V_{X,\beta}/n_X}{V_{R,\beta}/n_R} = \frac{L_X/n_X}{L_R/n_R} = \frac{L_X/m_X}{L_R/m_R} \tag{6}$$

The last term in eqn. 6 is of considerable practical value as it expresses $D_{X,R}$ directly from experimental data obtained by analysing one solution containing species X and R the molarities of which are m_X and m_R, respectively, and by evaluating the zone lengths, L_X and L_R, recorded. By combining eqns. 5 and 6 we obtain

$$L_X = \frac{b}{I \cdot v_{a,\beta} - H} \cdot d_R \cdot D_{X,R} \cdot n_X \tag{7}$$

Assuming one analytical run with a sample, for fixed reference species R and at constant working conditions (I, a, H), eqn. 7 can be written in a simplified form:

$$L_X = C \cdot D_{X,R} \cdot n_X \tag{8}$$

where C represents an apparatus constant. Hence, it can be seen that the ratios $L_X/D_{X,R}$ are directly proportional to the molar amounts of the respective species and represent directly the composition of a sample in arbitrary molar units. The method of converting the arbitrary molar amounts into the absolute molar amounts is then given by the working technique used for the quantitation. The most important quantitation technique based on the concept of correction factors is the internal standard technique.

Internal standard technique

To a defined sample volume, V_X, containing species X, the molarity m_X of which is to be determined, we add a defined volume, V_S, of a solution of a suitable standard species S the concentration of which in the solution is m_S. By this procedure, there is obtained a mixture in which the concentration of the species X, m_X', is $m_X V_X/(V_X + V_S)$ and of the species S, m_S', is $m_S V_S/(V_X + V_S)$. After injecting a suitable amount of the mixture, we evaluate zone lengths L_X' and L_S' in the isotachopherogram obtained. The unknown concentration of the species X in the sample is obtained by inserting m_X' and m_S' into eqn. 6;

$$m_X = \frac{L_X'}{L_S'} \cdot \frac{D_{S,R}}{D_{X,R}} \cdot \frac{V_S}{V_X} \cdot m_S \tag{9}$$

A considerable advantage of this technique is that the amount of the sample injected need not be known and, further, that both the species under analysis and the standard species are separated during one run. The only calibration quantities required for the quantitative interpretation are the relative correction factors, $D_{X,R}$ and $D_{S,R}$.

Determination of relative correction factors and the validity of their values under variable working conditions

The determination of $D_{X,R}$ follows directly from eqn. 6. In practice, it represents

an analysis of a known mixture of both the species X and R. For investigating the possibility of generalising $D_{X,R}$ values, eqn. 6 is combined with eqn. 1, to give

$$D_{X,R} = \frac{c_{R,\varrho}}{c_{X,\beta}} \tag{10}$$

As explained previously, the concentrations of ionic species in their respective zones are uniquely determined by the composition of the leading electrolyte and are independent of the electric current and hydrodynamic counterflow. Hence, it can be seen that this statement is also valid for the $D_{X,R}$ values. Thus, the value of $D_{X,R}$ is a characteristic constant of species X if all concentrations of the species present in the leading electrolyte are fixed. However, as $D_{X,R}$ is a dimensionless quantity, the presence of some degrees of freedom may be expected in defining the composition of the leading electrolyte. If we consider a typical isotachophoretic separation of species X and R in aqueous medium with buffering counterionic species B, where the system is well buffered and free H^+ and OH^- ions do not appreciably contribute to the electrical conductivity of the system, then the specific conductivity, κ_β, of the zone β containing species X is $\kappa_\beta = c_{X,\beta} \cdot u_{X,\beta} + c_{B,\beta} \cdot u_{B,\beta}$, where $u_{X,\beta}$ and $u_{B,\beta}$ are the net mobilities of species X and B in the zone β, respectively. Similarly, the specific conductivity, $\kappa_\varrho = c_{R,\varrho} \cdot u_{R,\varrho} + c_{B,\varrho} \cdot u_{B,\varrho}$, where the meaning of the symbols is evident. As the system is assumed to be well buffered, the pH values in the zones β and ϱ are constants corresponding to the acid–base equilibria of the species X and R with the buffering species B, respectively. This situation can be expressed by the relationships $c_{B,\beta}/c_{X,\beta} = k_{BX}$ and $c_{B,\varrho}/c_{R,\varrho} = k_{BR}$, where k_{BX} and k_{BR} are characteristic constants for species X and R and counterion B. By combining all previous relationships with eqns. 3 and 10, we obtain

$$D_{X,R} = \frac{u_{R,\varrho}}{u_{R,\varrho} + k_{BR} \cdot u_{B,\varrho}} \cdot \frac{u_{X,\beta} + k_{BX} \cdot u_{B,\beta}}{u_{X,\beta}} \tag{11}$$

It can be seen that at constant pH values in the zones (which implies constant mobilities) and for a given counterionic species B, the $D_{X,R}$ value is a characteristic constant of the species X for a fixed reference species R. As the pH values in the zones of the species separated are governed by the pH value in the leading zone α, the above assumptions can be related to the leading zone. Therefore, we can express the $D_{X,R}$ value by the following relationship:

$$D_{X,R}(B, \mathrm{pH}_\alpha) = \text{constant} \tag{12}$$

where the symbols in parentheses represent quantities that should be kept constant. This relationship has a considerable practical meaning as it gives a complete description of the working conditions that must be fixed in order that the $D_{X,R}$ value should be a characteristic quantitative constant of the species X. Therefore, if the counterionic species B is chosen and the pH in the leading electrolyte is kept constant, the values of $D_{X,R}$, once having been determined, can be used for the quantitative interpretation of any isotachopherogram, regardless of the electric driving current, hydrodynamic counterflow and the mobility and concentration of the leading ionic species used.

EXPERIMENTAL

The values of $D_{X,R}$ were measured for some model acid anions chosen from the metabolic cycle of citric acid, with the use of acetate as the reference ionic species, by analysing 3–5 μl of model mixtures in which the concentrations of the species in question were in the range 0.004–0.007 *M*. Analyses were carried out on a capillary apparatus[11,12], equipped with thermocouple detector[2] and with a PTFE capillary 30 cm long and 0.45 mm I.D. serving as the column. The column was maintained thermostatically at 23.5° and the electric driving current was stabilised within the range 70–100 μA. Four different leading electrolytes were used: 0.0137 *M* and 0.011 *M* hydrochloric acid, 0.0137 *M* nitric acid and 0.0081 *M* sulphuric acid. The pH was adjusted to 5.2 by adding urotropin, which served as the counterionic species. The terminating electrolyte was 0.01 *M* glutamic acid.

RESULTS AND DISCUSSION

Experimental values of the relative correction factors $D_{X,Ac}$ (Ac = acetate) are given in Table I. The values represent arithmetic means of five measurements. The value

TABLE I

RELATIVE CORRECTION FACTORS $D_{X,R}$ (R = ACETATE)

Ionic species, X	*Leading ionic species*				*Total average*
	0.0137 M chloride	*0.0137 M nitrate*	*0.0081 M sulphate*	*0.0110 M chloride*	
Citrate	2.24 ± 0.02	2.22 ± 0.02	2.23 ± 0.02	2.21 ± 0.02	2.225
Succinate	1.79 ± 0.01	1.79 ± 0.01	1.80 ± 0.01	1.79 ± 0.01	1.793
Fumarate	1.84 ± 0.02	1.84 ± 0.02	1.87 ± 0.02	1.87 ± 0.02	1.855
Malate	1.91 ± 0.02	1.92 ± 0.02	1.91 ± 0.02	1.90 ± 0.02	1.910
α-Ketoglutarate	1.91 ± 0.01	1.89 ± 0.02	1.91 ± 0.02	1.92 ± 0.02	1.908
Oxalate	1.72 ± 0.02	1.72 ± 0.02	–*	1.71 ± 0.02	1.717

* Value omitted; for explanation, see text.

of $D_{X,Ac}$ for oxalate is omitted for the case when sulphate was used as the leading ionic species because the difference in the mobilities of oxalate and sulphate was too low to be distinguished as completely resolved zones in the isotachopherogram. The precision of the given values, expressed as the relative width of the reliability interval for a confidence level of 95%, is approximately ± 1% (relative).

It can be seen that the total average of $D_{X,Ac}$ for a given species X is always within the respective confidence limits for all leading ionic species used. This means that the effect of different leading electrolytes on the $D_{X,Ac}$ values is really insignificant. Therefore, for a given ionic species, the $D_{X,R}$ values can be considered to be fairly good constants, irrespective of the concentration and mobility of the leading ionic species used, as was theoretically predicted.

CONCLUSIONS

To summarize the features of the concept of relative correction factors, it can be said that once the $D_{X,R}$ values have been determined by calibration under given working conditions, they can be used for the quantitative interpretation of isotachopherograms obtained under considerably different working conditions. When employing the internal standard technique, based on the concept of relative correction factors, $D_{X,R}$, calibration can be carried out once under given conditions and then quantitative analyses can be carried out by using any suitable leading ionic species and its concentration, at a different electric driving current and hydrodynamic counterflow, and, moreover, the amount of the sample injected need not be defined.

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