Journal of Chromatography, 390 (1987) 297–306
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 19 257

LIQUID CHROMATOGRAPHIC ISOLATION OF VINCRISTINE AND VINBLASTINE

DENIS DRAPEAU, HARVEY W. BLANCH* and CHARLES R. WILKE
Department of Chemical Engineering, University of California, Berkeley, CA 94720 (U.S.A.)
(First received September 1st, 1986; revised manuscript received November 11th, 1986)

SUMMARY

A fundamentally new method is described for the separation of the dimeric indole alkaloids vincristine and vinblastine from monomeric indole alkaloid impurities. This method uses an RP-18 high-performance liquid chromatography column with a methanol–water mobile phase containing an inorganic acid and an unusually low concentration of inorganic buffer. By keeping the buffer concentration low, the elution of all indole alkaloids is retarded, but the dimeric ones are retarded more than the monomeric ones. A theoretical model developed to explain this behavior postulates that the anions of the buffer solubilize the protonated indole alkaloids by pairing with them. Lowering the buffer concentration reduces the availability of pairing ions and thus decreases the mobile phase affinity of protonated alkaloids, particularly those having a 2+ charge. A similar approach may be applicable in other situations where ionogenic organic compounds having a particular valence must be separated from related compounds having different valences, or from non-ionogenic compounds.

INTRODUCTION

For either analysis or preparation of the anti-cancer agents vincristine and vinblastine, these dimeric indole alkaloids must be separated from the more than 90 monomeric indole alkaloids that accompany them in *Catharanthus roseus* leaf extracts. Some of these monomeric impurities are present at concentrations 100-fold higher than either vincristine or vinblastine. Most are very similar to vincristine and vinblastine in terms of both alkalinity and content of polar groups (Fig. 1) and therefore can not be distinctly separated from them by the standard liquid chromatographic (LC) techniques, which use reversed-phase[1–5], silica[6]. or alumina[7,8] packings.

This paper describes a reversed-phase high-peformance liquid chromatography (HPLC) technique that provides a clean separation of dimeric indole alkaloids from monomeric indole alkaloids. Vincristine and vinblastine, though minor components of *C. roseus* leaf extracts, are major components of the dimeric fraction and can therefore be isolated from this fraction by the standard LC techniques. The technique

0021-9673/87/$03.50

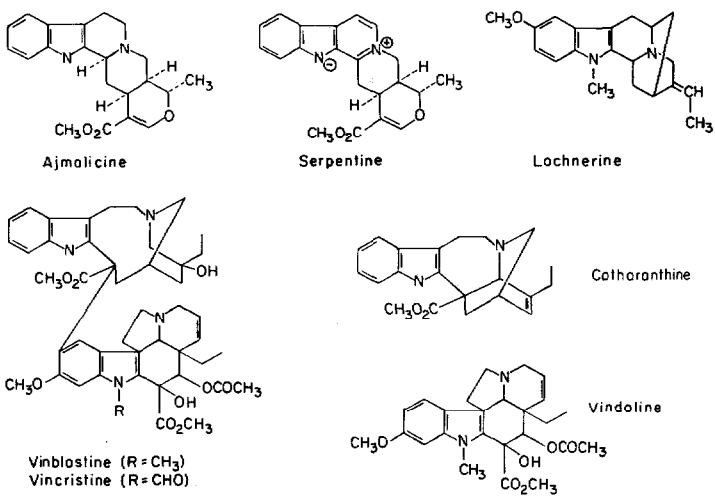

Fig. 1. Chemical structures of five monomeric and two dimeric indole alkaloids.

uses an acidic methanol–water mobile phase having a very low buffer concentration. The low buffer level retards the elution of all indole alkaloids, but particularly the dimeric ones. Extensive tailing of the individual peaks occurs, but this does not interfere with the isolation of dimeric indole alkaloids as a group.

This approach represents an alternative to size-exclusion chromatography for the separation of dimeric indole alkaloids from monomeric indole alkaloids[8].

EXPERIMENTAL

A Perkin-Elmer Series 2 HPLC system equipped with a Rheodyne 7105 injector was used. Two 25 cm × 4.6 mm I.D. EM Hibar II columns were used, one containing 10-μm LiChrosorb RP-18 particles and the other containing 10-μm LiChrospher SI-100 particles. Detection was performed with a Perkin-Elmer LC-85 variable-wavelength spectrophotometric detector set at 222 nm and a Kratos FS970 LC Fluorimeter set at 226 nm excitation and using a 340-nm cut-off filter.

Ajmalicine, vincristine, and vinblastine were obtained from Sigma. Serpentine tartrate was kindly provided by J. Berlin of the Gesellschaft für Biotechnologische Forschung mbH (Braunschweig, F.R.G.). Lochnerine, vindoline, and catharanthine were kindly provided by G. Cullinan and W. R. Fields of Eli Lilly (Indianapolis, IN, U.S.A.).

Mobile phase solvents were prepared using spectrophotometric grade methanol, water (distilled and deionized), dipotassium hydrogen phosphate, potassium dihydrogen phosphate, orthophosphoric acid, potassium acetate, acetic acid, ammonium dihydrogen phosphate, and sulfuric acid. Concentrations are expressed relative to final volume rather than relative to volume of water. Flow-rate was at 3 ml/min.

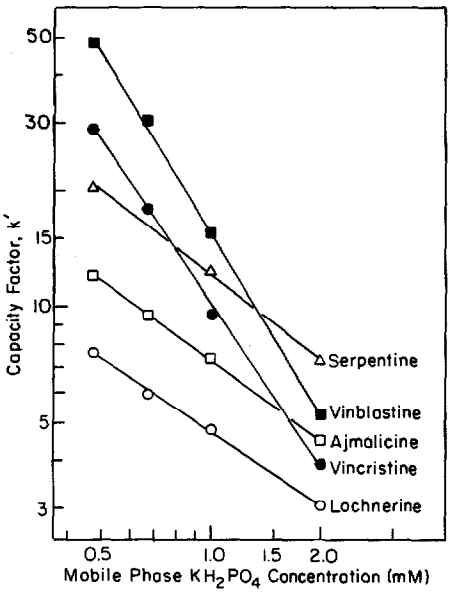

Fig. 2. Influence of mobile phase KH_2PO_4 concentration on retention of lochnerine, ajmalicine, serpentine, vincristine, and vinblastine by RP-18 packing. Mobile phase consists of phosphoric acid at 3.0 m*M* in methanol–water (85:15) with KH_2PO_4 at specified concentrations.

RESULTS

The effect of mobile phase KH_2PO_4 concentration on the capacity factors of several indole alkaloids is shown in Fig. 2. As KH_2PO_4 concentration is lowered from 2.0 m*M* to 0.47 m*M*, the capacity factor (defined in the table of nomenclature)

TABLE I

INFLUENCE OF MOBILE PHASE KH_2PO_4 CONCENTRATION ON RETENTION OF FIVE MONOMERIC INDOLE ALKALOIDS AND TWO DIMERIC INDOLE ALKALOIDS BY RP-18 PACKING

Mobile phase contains specified concentrations of phosphoric acid and KH_2PO_4 in methanol–water (85:15).

Alkaloid	*Capacity factor, k'*		$\frac{\Delta \ln k'}{\Delta \ln [KH_2PO_4]}$
	$[KH_2PO_4] = 2.00\ mM$ $[H_3PO_4] = 3.0\ mM$	$[KH_2PO_4] = 0.47\ mM$ $[H_3PO_4] = 3.0\ mM$	
Lochnerine	2.05	6.6	−0.81
Ajmalicine	3.48	11.0	−0.80
Catharanthine	3.59	11.3	−0.79
Vindoline	3.68	11.1	−0.76
Serpentine	6.17	19.5	−0.80
Vincristine	2.96	28.1	−1.55
Vinblastine	4.28	47.4	−1.66

TABLE II

INFLUENCE OF MOBILE PHASE $NH_4H_2PO_4$ CONCENTRATION ON RETENTION OF THREE MONOMERIC INDOLE ALKALOIDS AND ONE DIMERIC INDOLE ALKALOIDS BY RP-18 PACKING

Mobile phase contains specified concentrations of phosphoric acid and $NH_4H_2PO_4$ in methanol–water (85:15).

Alkaloid	*Capacity factor, k'*		$\frac{\Delta \ln k'}{\Delta \ln [NH_4H_2PO_4]}$
	$[NH_4H_2PO_4] = 2.00\ mM$ $[H_3PO_4] = 0.60\ mM$	$[NH_4H_2PO_4] = 0.80\ mM$ $[H_3PO_4] = 0.36\ mM$	
Lochnerine	3.38	7.57	−0.88
Ajmalicine	6.04	13.45	−0.87
Catharanthine	6.64	14.94	−0.89
Vincristine	4.54	16.59	−1.41

increases by a factor of 9 or more for dimeric indole alkaloids but only by a factor of 3 for monomeric indole alkaloids (Table I). Similar behavior is seen when ammonium dihydrogen phosphate is substituted for KH_2PO_4 (Table II).

The effect of KH_2PO_4 concentration is not simply due to its influence on mobile phase pH. The pH is governed by the ratio of phosphoric acid activity and KH_2PO_4 activity and thus responds to changes in the concentrations of either of these solutes. While lowering the KH_2PO_4 concentration retards elution of ajmalicine and lochnerine (Fig. 2), raising the phosphoric acid concentration does not (Fig. 3). Switching to an extremely acidic mobile phase [150 m*M* sulfuric acid in methanol–water (85:15)] actually accelerates the elution of indole alkaloids; capacity factors of

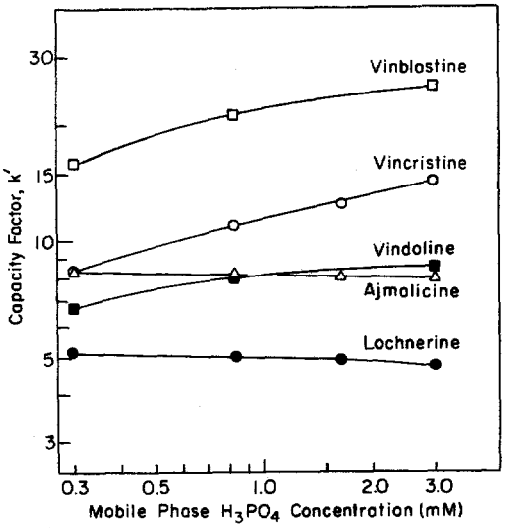

Fig. 3. Influence of mobile phase phosphoric acid concentration on retention of lochnerine, ajmalicine, vindoline, vincristine, and vinblastine by RP-18 packing. Mobile phase consists of KH_2PO_4 at 0.70 m*M* in methanol–water (85:15) with phosphoric acid at specified concentrations.

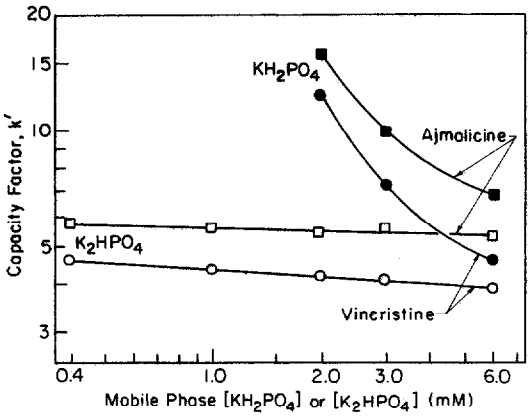

Fig. 4. Influence of mobile phase KH_2PO_4 or K_2HPO_4 concentration on retention of ajmalicine and vincristine by RP-18 packing. Mobile phase consists of methanol–water (70:30) containing KH_2PO_4 or K_2HPO_4 at specified concentrations.

all seven indole alkaloids listed in Table I were reduced to less than 2.0 by this mobile phase.

Instead, the effect of KH_2PO_4 concentration appears to be associated with its role in providing anions that can pair with alkaloid cations. The phosphoric acid concentration has little effect on anion concentration since it is only slightly dissociated at the low dielectric constant of methanol–water (85:15). KH_2PO_4, however, is probably fully dissociated into K^+ and $H_2PO_4^-$ ions.

The influence of anion concentration on alkaloid retention depends on the alkaloids being in their protonated forms. This is evident in Fig. 4. When the mobile phase contains K_2HPO_4 rather than KH_2PO_4, ajmalicine is primarily in the non-protonated form, since the HPO_4^{2-} ion is more basic (pK_a = 7.2) than ajmalicine (pK_a = 6.3). Consequently ajmalicine retention is unaffected by the mobile phase anion concentration. Vincristine, which has one site that is more alkaline (pK_a =

TABLE III

INFLUENCE OF MOBILE PHASE POTASSIUM ACETATE CONCENTRATION ON RETENTION OF THREE MONOMERIC INDOLE ALKALOIDS AND TWO DIMERIC INDOLE ALKALOIDS BY RP-18 PACKING

Mobile phase contains specified concentrations of acetic acid and potassium acetate in methanol–water (85:15).

Alkaloid	*Capacity factor, k'*		$\frac{\Delta \ln k'}{\Delta \ln [CH_3COOK]}$
	[CH₃COOK] = 1.00 mM *[CH₃COOH] = 1.0 mM*	*[CH₃COOK] = 0.60 mM* *[CH₃COOH] = 1.0 mM*	
Ajmalicine	3.62	6.20	−1.05
Catharanthine	5.94	9.54	−0.93
Vindoline	1.68	2.30	−0.62
Vincristine	3.57	6.03	−1.03
Vinblastine	5.09	8.70	−1.05

7.4) than ajmalicine, is partially protonated in the presence of HPO_4^{2-}, so its retention is somewhat affected by anion concentration.

The reason that mobile phase anion concentration affects dimeric indole alkaloids differently than monomeric indole alkaloids is that in the presence of phosphoric acid alkaloids of the former group are protonated at two locations and thus form divalent cations. When acetic acid (pK_a = 4.75) is substituted for phosphoric acid (pK_a = 2.1), vincristine behaves similarly to ajmalicine (Table III). In the presence of acetic acid, the less alkaline site (pK_a = 5.0) on the vincristine molecule apparently has a low degree of protonation. Vinblastine, vincristine, and vindoline (pK_a = 5.5) apparently are not fully protonated even by phosphoric acid until the acid concentration reaches roughly 3.0 m*M* (Fig. 3).

To investigate whether the silica support material of the RP-18 packing plays a role, a non-derivatized silica packing was substituted. Although this gave much lower capacity factors, the effect of anion concentration was similar (Table IV).

THEORETICAL MODEL

The observed behavior can be explained by postulating that the alkaloid ions in the mobile phase are predominantly in the form of ion pairs whereas those at the surface are predominantly unpaired. Under such conditions a decrease in the concentration of the pairing ion causes the partitioning to shift away from dissolved ion pairs and toward adsorbed ions.

The partitioning can be expressed quantitatively by

$$k' = \frac{V_s}{V_m} \frac{[H_zB^{z+}]_s}{[H_zB^{z+} \cdot zA^-]_m} \tag{1}$$

where k' is the capacity factor, V_s/V_m is the volume ratio of the stationary (s) and

TABLE IV

INFLUENCE OF MOBILE PHASE KH_2PO_4 CONCENTRATION ON RETENTION OF FIVE MONOMERIC INDOLE ALKALOIDS AND TWO DIMERIC INDOLE ALKALOIDS BY SILICA PACKING

Mobile phase contains specified concentrations of phosphoric acid and KH_2PO_4 in methanol–water (85:15).

Alkaloid	*Capacity factor, k'*		$\frac{\Delta \ln k'}{\Delta \ln [KH_2PO_4]}$
	$[KH_2PO_4]$ = 1.00 m*M* $[H_3PO_4]$ = 0.3 m*M*	$[KH_2PO_4]$ = 0.20 m*M* $[H_3PO_4]$ = 0.3 m*M*	
Lochnerine	1.85	4.64	−0.57
Ajmalicine	1.97	5.10	−0.59
Catharanthine	1.98	4.97	−0.57
Vindoline	1.73	4.75	−0.63
Serpentine	2.53	6.12	−0.55
Vincristine	1.97	7.92	−0.86
Vinblastine	2.27	12.72	−1.07

mobile (m) phases, $[H_zB^{z+}]_s$ is the surface phase concentration of an unpaired z-valent alkaloid ion, and $[H_zB^{z+} \cdot zA^-]_m$ is the mobile phase concentration of the same ion paired with z monovalent A^- ions.

In order to predict the effect of the A^- concentration $[A^-]_m$ on k', the thermodynamic relationships linking $[H_zB^{z+}]_s$ to $[H_zB^{z+} \cdot zA^-]_m$ must be used. These relationships are

$$(H_zB^{z+} \cdot zA^-)_m \overset{K_1}{\rightleftarrows} (H_zB^{z+})_m + z(A^-)_m \tag{2}$$

and

$$(H_zB^{z+})_m \overset{K_2}{\rightleftarrows} (H_zB^{z+})_s \tag{3}$$

which can be expressed as

$$[H_zB^{z+} \cdot zA^-]_m = \frac{1}{K_1} \frac{\gamma(H_zB^{z+})_m \cdot [H_zB^{z+}]_m \cdot \{\gamma(A^-)_m\}^z \cdot [A^-]_m^z}{\gamma(H_zB^{z+} \cdot zA^-)_m} \tag{4}$$

and

$$[H_zB^{z+}]_s = K_2 \frac{\gamma(H_zB^{z+})_m \cdot [H_zB^{z+}]_m}{\gamma(H_zB^{z+})_s} \tag{5}$$

where k_1 and K_2 are equilibrium constants, $\gamma(H_zB^{z+} \cdot zA^-)$, $\gamma(H_zB^{z+})$ and $\gamma(A^-)$ are the activity coefficients of the paired alkaloid ion, the unpaired form of the alkaloid ion and the paired ion, respectively. Substitution of eqn. 4 and 5 into eqn. 1 gives

$$k' = \frac{V_s K_1 K_2}{V_m} \frac{\gamma(H_zB^{z+} \cdot zA^-)_m}{\gamma(H_zB^{z+})_s \cdot \{\gamma(A^-)_m\}^z \cdot [A^-]_m^z} \tag{6}$$

Since a linear relationship between $\ln k'$ and $\ln [A^-]_m$ has been observed (Fig. 1), eqn. 6 is more conveniently expressed in logarithmic form:

$$\ln k' = \ln \left\{ \frac{V_s K_1 K_2}{V_m} \gamma(H_zB^{z+} \cdot zA^-)_m \right\} + \\ - \ln \gamma(H_zB^{z+})_s - z \ln \gamma(A^-)_m - z \ln [A^-]_m \tag{7}$$

The first term on the right-hand side of eqn. 7 consists of variables that are all only weakly dependent on $[A^-]_m$ as discussed by Horváth *et al.*[9]. Therefore

$$\frac{\partial \ln k'}{\partial \ln [A^-]_m} \approx - \frac{\partial \ln \gamma(H_zB^{z+})_s}{\partial \ln [A^-]_m} - z \frac{\partial \ln \gamma(A^-)_m}{\partial \ln [A^-]_m} - z \tag{8}$$

The second term on the right-hand side of eqn. 8 can be estimated using the Debye–Huckel law[10]:

$$\ln \gamma(A^-)_m = -25.8 \cdot [A^-]_m^{0.5} \cdot \varepsilon_m^{-1.5} \quad (9)$$

where $[A^-]_m$ is expressed in mM and ε_m is the dielectric constant. Using $\varepsilon_m = 32.7$ (pure methanol) gives

$$\ln \gamma(A^-)_m = -0.138 \cdot [A^-]_m^{0.5} \quad (10)$$

which leads to

$$\frac{\partial \ln \gamma(A^-)_m}{\partial \ln [A^-]_m} = -0.069 \cdot [A^-]_m^{0.5} \quad (11)$$

The first term on the right-hand side of eqn. 8 can also be estimated using the Debye–Huckel relationship:

$$\ln \gamma(H_zB^{z+})_s = -25.8 \cdot z^2 \cdot [A^-]_m^{0.5} \cdot \varepsilon_s^{-1.5} \quad (12)$$

Although this activity coefficient applies at the interface, the pertinent ionic strength is that of the mobile phase $[A^-]_m$ because the Debye length[10] at $[A^-] = 1$ mM is 62 Å, which is far greater than the thickness of either the surface layer or the silica support material[11]. The dielectric constant ε_s will be influenced by the C_{18} chains and the silica support, but a reasonable approximation is again that of pure methanol. Thus

$$\frac{\partial \ln \gamma(H_zB^{z+})_s}{\partial \ln [A^-]_m} = -0.069 \cdot z^2 \cdot [A^-]_m^{0.5} \quad (13)$$

Substitution of eqns. 11 and 13 into eqn. 8 gives

$$\frac{\partial \ln k'}{\partial \ln [A^-]_m} = -z + 0.069 \cdot (z^2 + z) \cdot [A^-]_m^{0.5} \quad (14)$$

which in the vicinity of $[A^-]_m = 1.0$ mM becomes

$$\frac{\partial \ln k'}{\partial \ln [A^-]_m} \approx -z + 0.069 \cdot (z^2 + z) \quad (15a)$$

$$\approx -0.86 \text{ for } z = 1 \quad (15b)$$

$$\approx -1.66 \text{ for } z = 2 \quad (15c)$$

The agreement with data in Tables I and II is excellent.

The assumption on which this model is based (that paired ions predominate in the mobile phase while unpaired ions predominate in the surface phase was chosen strictly because it fits the data. If instead we had assumed that unpaired ions were predominant in the mobile phase and paired ions were predominant at the surface, the predicted values of $(\partial \ln k')/(\partial \ln [A^-]_m)$ would have been positive. Alternatively, if we had assumed that either unpaired ions were predominant in both phases or paired ions were predominant in both phases, the predicted values of $(\partial \ln k')/(\partial \ln$

$[A^-]_m$) would have been of much smaller absolute value than those listed in Tables 1–4.

This model is supported by: (1) the known tendency of ions to pair in liquids having dielectric constants substantially lower than that of water, and (2) the known protophilic nature of silica. Protonated alkaloid ions may be stabilized at the surface simply by their proximity to the silica support material (which will be well within one Debye length of them) while in the mobile phase they may require pairing with protophilic ions such as $H_2PO_4^-$ or CH_3COO^- for stability. Adsorption of alkaloid ions may be accompanied by release of protons from silicic acid groups and thus may be a form of ion exchange.

DISCUSSION

A mobile phase consisting of methanol–water (85:15) with 3 m*M* phosphoric acid and KH_2PO_4 at 0.5 m*M* will elute the dimeric indole alkaloids from an RP-18 packing substantially later than the monomeric indole alkaloids. If *C. roseus* leaf extracts are injected, the monomeric indole alkaloids will be eluted first leaving only the dimeric indole alkaloids, including vincristine and vinblastine, on the column. The dimeric fraction can then be eluted quickly by increasing either the KH_2PO_4 concentration or the pH of the mobile phase. Vincristine and vinblastine can be isolated from this fraction by any of the standard LC techniques[1–8]. Alternatively the leaf extracts can be subjected first to one of the standard techniques to obtain impure vincristine and vinblastine fractions that can then each be purified using the approach described here.

The use of low buffer strength appears to be a novel approach that may be applied in other cases where ionogenic organic compounds having a particular valence must be separated from similar compounds having different valences, or from non-ionogenic compounds. The critical characteristics of the mobile phase appear to be the high proportion of organic solvent, the presence of an inorganic acid (or base if the organic ions to be separated are anions), and the low concentration of inorganic counterions.

ACKNOWLEDGEMENTS

The authors wish to thank Aldo F. Sciamanna for valuable advice regarding experimental procedures. This work was supported by the Center for Biotechnology Research (San Francisco, CA, U.S.A.).

REFERENCES

1 S. Görög, B. Herényi and K. Jovánovics, *J. Chromatogr.*, 139 (1977) 203.
2 M. Verzele, L. De Taeye, J. Van Dyck, G. De Decker and C. De Pauw, *J. Chromatogr.*, 214 (1981) 95.
3 J.-P. Renaudin, *J. Chromatogr.*, 291 (1984) 165.
4 M. De Smet, S. J. P. Van Belle, G. A. Storme and D. L. Massart, *J. Chromatogr.*, 345 (1985) 309.
5 J. E. Bodner, J. R. Chen, W. H. Johns, E. P. Mariani and E. C. Shinal, *J. Pharm. Sci.*, 72 (1983) 535.
6 E. Leverd, D. Beziat and Ph. Hatinguais, *Boll. Chim. Farm.*, 117 (1978) 27.
7 K. Jovanovics, E. Bittner, E. Dezseri, J. Eles and K. Szasz, *Ger. Pat.*, 2,124,023 (1971).

8 W. E. Jones, *Ger. Pat.*, 2,442,245 (1975).
9 Cs. Horváth, W. Melander and I. Molnar, *Anal. Chem.*, 49 (1977) 142.
10 G. W. Castellan, *Physical Chemistry*, Addison-Wesley, Reading, MA, 2nd ed., 1971, pp. 370–377.
11 M. Verzele, C. DeWaele and D. Duquet, *J. Chromatogr.*, 329 (1985) 351.