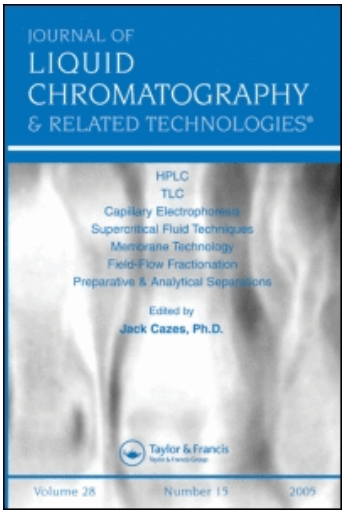

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ON THE MEASUREMENT OF DEAD TIME IN MICELLAR LIQUID CHROMATOGRAPHY

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ABSTRACT

Modelling of the retention of solutes in micellar liquid chromatography allows the optimization of the resolution of a mixture of solutes and the determination of physico-chemical retention parameters. Both tasks imply the calculation of capacity factors, which are severely affected by the value of dead time. However, the determination of the dead time is not easy when a micellar mobile phase is used owing to the wide and variable perturbations that appear at the heads of the chromatograms. Four different criteria of determination of a reference time in the chromatograms are proposed and compared. The criteria are applied to mobile phases containing a varying concentration of surfactant and modifier, in order to observe the dependence of the reference time with the mobile phase composition. The study of the influence of the errors in the dead time on the modelling of the retention indicated that an accurate dead time is necessary to calculate retention parameters, but an excellent prediction of retention times can be achieved with a wide range of values of dead time.

INTRODUCTION

A growing interest is being paid to the use of micellar eluents in reverse phase liquid chromatography. Among the procedures reported in micellar liquid chromatography (MLC), the applications to the determination of drugs in physiological samples have a particular attraction.[1-6] Mobile phases containing a surfactant above the critical micellar concentration solubilize the proteins in these samples, avoiding thus the long previous separation steps which are often necessary in the conventional procedures. Owing to the diverse types of possible interactions (e.g., ionic, hydrophobic and steric) between solute, mobile phase and stationary phase, compounds of different character can be separated in a mixture when eluted with micellar eluents. Although in the first reports these eluents only contained a surfactant solution, most of the published procedures have used mobile phases of surfactant containing an organic modifier, usually an alcohol. The presence of the modifier is required since it usually increases the efficiency of the chromatographic peaks and allows a better control of the eluent strength.

In previous works we reported the possibility of modelling the retention behaviour of solutes in MLC.[7-10] An accurate modelling of retention is necessary in order to adequately optimize the separation of a mixture of compounds, especially when the efficiencies achieved are rather low. The retention models relate the capacity factor, k', with the composition of the eluent, thus avoiding the dependence of the model with the flow-rate.

The capacity factor is defined as:

$$k' = \frac{t_R - t_0}{t_0} \qquad (1)$$

where t_R is the retention time and t_0 the dead time. The relationship between k' and the micellar concentration, [M], in the absence of any modifier, has been theoretically derived according to several approaches:[11-13]

$$k' = \frac{K_{SW}}{1 + K_{AM}\,[M]} \qquad (2)$$

Elution of a solution in MLC depends on two partition equilibria: one between the stationary phase and water (K_{SW}), and the other inside the mobile phase between water and the micelle (K_{AM}). The constant K_{SW} is the ratio of the volume of the stationary phase to the volume of the mobile phase in the column (ϕ), multiplied by the partition coefficient between the stationary phase and water (P_{SW}). This

equation has been verified for many solutes, and the solute-micelle association constant coincided with the value obtained by other non-chromatographic techniques.[12]

When an alcohol is added to the mobile phase, the retention model grows in complexity:[10]

$$k' = \frac{K_{SW}\,\dfrac{1+K_{SD}\varphi}{1+K_{AD}\varphi}}{1+K_{AM}\,\dfrac{1+K_{MD}\varphi}{1+K_{AD}\varphi}\,[M]} \qquad (3)$$

being φ the alcohol concentration (v/v). In this equation several new constants were introduced: K_{AD} indicates the displacement of the equilibria to release more analyte from the stationary phase and the micelle, towards the bulk water, in the presence of modifier, and K_{SD} and K_{MD} are correcting constants of K_{SW} and K_{AM}, respectively, which are necessary to take into account other interactions with the stationary phase and micelle. Equation (3) has also been checked for several groups of compounds, the errors achieved being usually below 2%.[10]

However, the capacity factor is very sensitive to errors in the value of dead time. The reliable determination of the dead time is especially difficult when the retention of a compound should be described in chromatographic systems where the nature of the mobile phase is largely modified. The change of eluent originates an important modification in the shape of the heads of the chromatograms.

In the MLC literature, the procedures described to measure the dead time made use of injection of water[14,15] salt solutions such as $NaNO_3$,[16,17] NaI and KI,[11,18] or organic solvents such as methanol[12,19] and acetonitrile.[20] The criterium applied to locate the dead time was usually the measurement of the position of the maximum of the first peak, or the measurement of the time from the injection to the first deviation from the base-line. Assignation of these times to the dead time only implies an approximation to reality, since: (i) the residence time of the mobile phase in the pre- and post-column conducts located between injector and detector is ignored, (ii) it is supposed that the injected compound used to determine the dead time does not interact with the stationary phase, and (iii) the wide and variable perturbation observed at the beginning of the chromatograms in MLC causes an uncertainty in the location of the point where the dead time should be measured. Therefore, it is questionable that the values given in the MLC literature were real dead times, although the measured times were probably close to them.

In this work, four different criteria of determination of a characteristic time in the head of the chromatograms, which will be called reference time, are proposed and compared. The criteria were applied to mobile phases containing a varying concentration of surfactant and modifier, in order to observe the dependence of the reference time with the mobile phase composition. The effect of the use of a reference time as dead time in the calculation of capacity factors to model the retention of diverse solutes is finally studied.

MATERIALS AND METHODS

Reagents

Sodium dodecyl sulphate (SDS), 1-butanol (Merck, Darmstadt, Germany), and 1-propanol (Panreac, Barcelona, Spain) were used to prepare the mobile phases and some injected solutions. The micellar mobile phases were prepared by mixing the aqueous surfactant solution with the alcohol to obtain the working concentration (v/v percentage). The mobile phases were buffered at pH 3 with citric acid monohydrate and NaOH (Panreac).

The solutes injected were diuretics: amiloride (ICI Farma, Madrid, Spain), bendroflumethiazide (Davur, Madrid), chlorthalidone (Ciba-Geigy, Barcelona), ethacrynic acid (Merck), spironolactone (Searle, Madrid), and triamterene (Sigma, Buchs, Switzerland). These compounds, except triamterene, were kindly donated by pharmaceutical laboratories located in Spain. Stock solutions were prepared in SDS micellar solutions after addition of a small volume of methanol to facilitate dissolution. Other compounds that were injected were KI (Probus, Barcelona), $NaNO_3$ (Panreac), acetonitrile (Merck) and methanol (Panreac). Barnstead nanopure, deionized water (Sybron, Boston, MA, USA) was used throughout.

Apparatus

Two Hewlett-Packard HP 1050 (Palo Alto, CA, USA) liquid chromatographs with isocratic pumps, UV-visible detectors and HP 3396A integrators were used. Most of the chromatograms were obtained with a system implemented with an autosampler. Data acquisition was performed through the PEAK-96 software (Hewlett-Packard, Avondale, PA, USA). The mobile phase flow-rate was 1 mL/min. A Spherisorb ODS-2 column (5 μm particle size, 125 mm x 4.6 mm I.D.) and precolumn (35 mm x 4.6 mm I.D.) (Scharlau, Barcelona) were used. The

mobile phase and the injected solutions were filtered through 0.45 μm and 0.22 μm Nylon membranes, respectively (Micron Separations, Westboro, MA, USA).

Data Treatment

The determination of the reference time used the following criteria:

Criterium 1: Search of the first main maximum or minimum

This procedure is the most commonly used in the MLC literature, where the appearance of the maximum or minimum of the first important perturbation in the chromatogram is searched. First, the region corresponding to the base-line (previous to the first perturbation, including time values from zero to t_b) is marked (Figure 1a), and fitted to a straight-line. Next, a second region is marked between t_{b+1} and t_p, being t_p a point following the perturbation, and the base-line is subtracted. After base-line correction, the maximum or minimum of the signal is searched in this region, by means of the bisection method. In order to improve the accuracy, a Lagrange interpolation is also applied to obtain the intermediate values.[21]

Frequently, an extra noise of different origin appears in the chromatograms, such as air bubbles, pump pressure fluctuations, modifications in the intensity of the light in the detector lamp, or even, alteration of the base-line due to previous injections. For this reason, in this criterium and in the criteria of formation of groups, a self-consistence cycle of the base-line has been included to eliminate anomalous points.

Criterium 2: Formation of groups scaling the noise

When the first alteration of the base-line appears, a simultaneous noise increase is produced. In this criterium, it is considered that the first perturbation begins when the noise exceeds a value that amounts the product of an arbitrary number, p, by the mean noise of the base-line, $\bar{n}_b$. The use of a constant value of p, previously established, may be problematic, since the noise may largely vary from one chromatogram to another. Thus, it was decided to scale the signal, by searching the highest value of the noise inside the region of interest (between t_{b+1} and t_p), n_{max}, and using as the minimum value of noise, $n_{min} = \bar{n}_b + 2.5\ \sigma_b$ (the factor 2.5 corresponds to a 98.8% probability), where σ_b is the standard deviation of the base-line noise. Finally, the noise levels were obtained by dividing the interval between n_{min} and n_{max} in a convenient number of equal divisions, N. Thus, the noise associated to level *l*, is given by:

$$n_l = n_{min} + \frac{1}{N}(n_{max} - n_{min}) \quad (4)$$

In this work, a value N = 300 has been arbitrarily taken.

The noise associated to each point in the region included between t_{b+1} and t_p was evaluated with the following expression:

$$n_i = \left| \frac{y_{i+1} + y_{i-1}}{2} - y_i \right| \quad (5)$$

where *y* is the value of the signal in arbitrary unities. This expression is proportional to the second derivative of the signal in the middle point of the interval. The mean noise of the base-line is obtained by:

$$n_b = \sum_{i=2}^{b} \frac{n_i}{b-1} \quad (6)$$

being b the last point taken in the region of the chromatogram considered as base-line.

Figure 1a shows a part of a chromatogram corresponding to the injection of a solution of similar composition to that of the mobile phase, which will be called blank solution. The formation of the peak is due to the unavoidable small difference in composition between the mobile phase and the injected solution, and to the presence of other reagents, such as citrate buffer.

The examination of each level always begins at time t_{b+1} . The noise in that point is measured and compared with the noise associated to the first level being established ($l = 1$ in equation 1). If the noise of the point is lower, it is rejected and the next point is taken. If the point fulfils the condition of having a noise larger than that of the examined level, the time of the point is assigned to the level, which for the first point will be t_{b+1} . The same sequence is followed with the next noise level, where the point that fulfilled the latter condition is first examined. The time of the first point that fulfils the condition of exceeding a given noise level is assigned to the level. The process is successively repeated until the maximum noise level (level 300) is reached. It is not necessary that the examination covers all the experimental points each time that a new level is studied, since a part of the chromatogram should be discarded after studying a previous noise level.

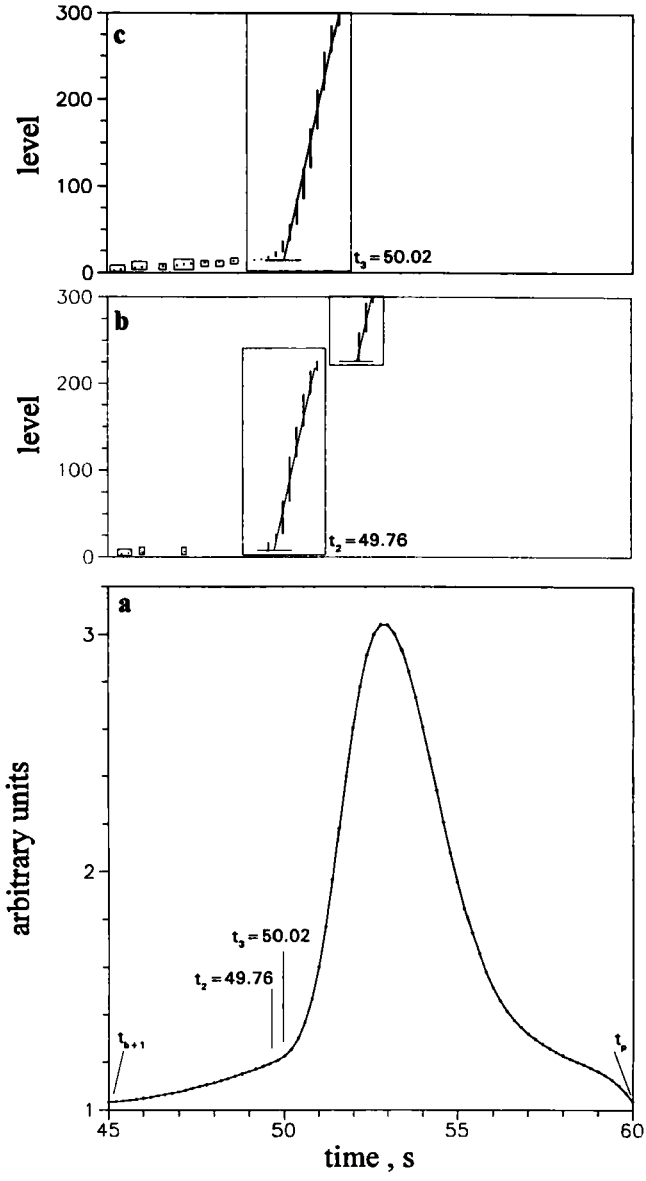

Figure 1. Methods of groups applied to the measurement of the reference time when a blank solution was injected into a 0.125 M SDS mobile phase containing 1.5% butanol. (a) Head of the chromatogram; (b) formation of groups scaling the noise; (c) formation of groups scaling the slope.

When the noise level is plotted vs. the time assigned to each level, a scaled diagram is observed (Figure 1b). Each vertical line is formed by the noise levels for which the same time has been assigned, that is, for which the same point in the chromatogram has exceeded for the first time the noise of the level. The magnitude of each vertical segment is related to the number of noise levels having the same time.

The formation of groups of lines with times corresponding to successive points in the chromatogram (separated in 0.2 s, owing to the rate of acquisition of the data by the chromatographic system) is observed in Figure 1b. The chromatogram in the figure involves five groups. The three first groups only include one or two points, whereas a large number of levels (about 220) belong to the fourth group, from 49.6 s to 51.0 s. The last group begins at 52.2 s and ends at 52.6 s, where the maximum noise level is reached.

Once the groups have been delimited, the points inside each group are fitted to a straight-line. The intersection of this line with the lowest noise level of the group will give a characteristic time. In this way, each group will be associated to a minimum value of the time, and one of these values will be taken as the reference time. The reference time should fulfil three conditions:

(i) It should belong to the base-line or should be close to it. This implies that it should be associated to a perturbation having an initial group appearing at the beginning of the chromatogram. Those groups including a very low number of levels should be rejected.

(ii) The importance of the perturbation is proportional to the number of levels of a group, therefore the reference time will be given by the group including more levels.

(iii) The reference time will be associated with an abrupt raise of the noise, consequently it will correspond to that group showing the largest slope.

Therefore, the reference time will be usually associated to that group showing a large and steep increase in the noise, starting from the base-line. If the three conditions are satisfied for a given group, the associated time is taken as the reference time. This situation is frequently encountered, but sometimes some disagreements exist, and the whole available information on the different groups should be examined in detail to select a time value.

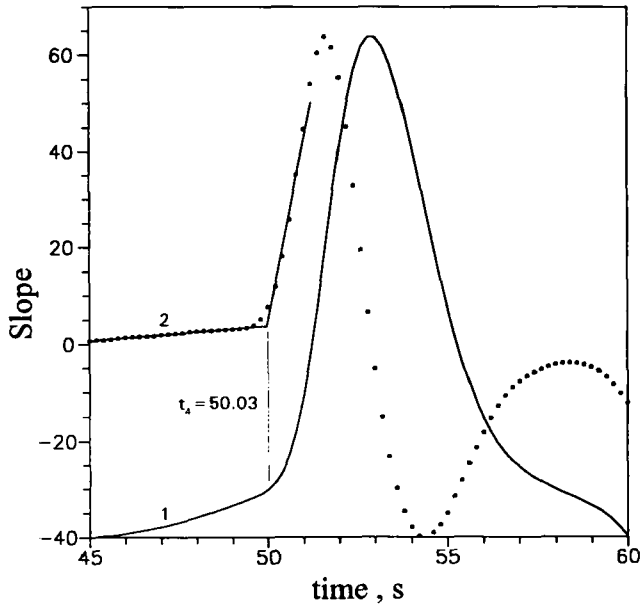

Figure 2. Method of the first derivative applied to the chromatogram shown in **Figure 1a.**

Criterium 3: Formation of groups scaling the slope

This criterium is a variant of criterium 2, where the slope around each point in the chromatogram is measured. The slope associated to a point *i* is measured by taking a set of five points around point *i*, and fitting the five points to a straight line. The slopes should be constant and with a low value in the base-line, and should change abruptly when the perturbation giving the reference time appears. To locate the reference time, the mean value and standard deviation of the slopes of the points belonging to the base-line are first obtained. Next, the slopes of the points in the marked region between t_{b+1} and t_p are measured, the largest slope is searched and the slope levels are scaled, similarly to the way indicated for the method described above to form the noise levels. Figure 1c shows the formation of eight groups, the main group being clearly the last one. This group contains the required information to measure the reference time.

Criterium 4: Method of the first derivative

This criterium is based on the detection of an important change in the slope of the base-line, when the significant perturbation is produced. The derivative of the chromatogram is calculated by measuring the slope associated to each point with a set of five points, as above (Figure 2). In this way, two regions with a well differentiated slope will appear at the beginning of the chromatogram. One of the
slopes will be nearly constant and low, and the other will increase or decrease largely. The values of slope in the proximity of the transition between both regions are plotted against time. Finally, the intersection of the two fitted straight-lines after rejecting the points in the intermediate curvature, will give the value of the reference time.

RESULTS AND DISCUSSION

Determination of the Reference Time

Figure 3 shows chromatograms corresponding to the injection of water and blank solution into a mobile phase of 0.1 M SDS/1.5% butanol, where the values of the reference time determined according to each one of the four criteria areindicated. Obviously, when the reference time was measured at the maximum of the peak (criterium 1), it was larger than the time given by the other three criteria. Criterium 1 also yielded different values for water and blank solution.

To study the nature of the signal obtained at the head of the chromatograms, several experiments were performed where water or a blank solution were injected, and the absorbance was measured at different wavelengths. It may be observed in Figure 4 that when water was injected, two well differentiated regions appeared at the beginning of the chromatogram, the first region being rather irregular and unpredictable, and the second region keeping its shape and position when the wavelength was changed. On the other hand, the signals obtained by injection of blank solution kept the position of the maximum. In this case, only the height of the signal was modified, being increased at decreasing wavelengths. The second perturbation was scarcely observed in this chromatogram. The noise increase observed with the wavelength, when water was injected, was probably associated to the state of the lamp and of the material of the detector cell. This noise was not so evident when the blank solution was injected owing to the wider range of the ordinate scale.

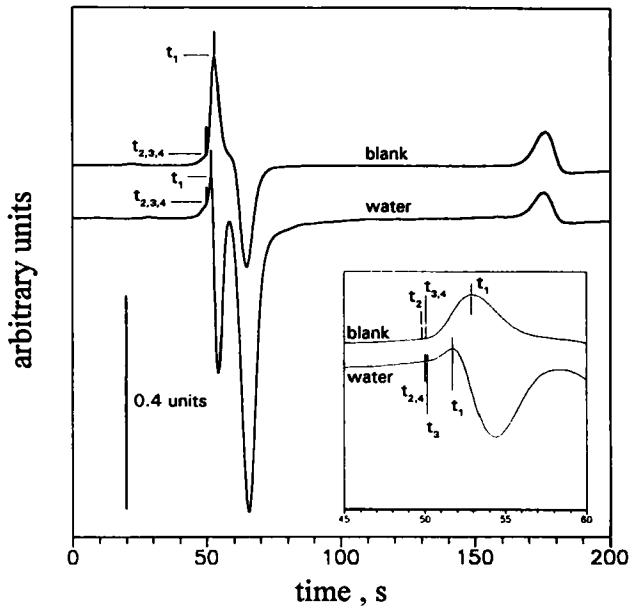

Figure 3. Heads of chromatograms corresponding to the injection of blank solution and water into a 0.125 M SDS/1.5% butanol mobile phase. The reference times indicated correspond to the maximum (t_1), formation of groups scaling the noise (t_2), formation of groups scaling the slope (t_3) and the method of the first derivative (t_4).

The first perturbation observed when water was injected had probably a refractometric origin and the second was an absorptiometric signal. The irregular shape of the first peak obtained with water was due to the large difference in composition between the injected solution and the mobile phase, which originated erratic fluctuations in the refraction index when both solutions were mixed. In contrast, when the blank solution was injected, the homogenization of the mixture was more simple. To verify this hypothesis, the concentration of SDS in the injected sample was varied in another series of experiences, from a value below the concentration of the mobile phase up to a concentration above it. In Figure 5, it may be observed that when the compositions of the injected solution and mobile phase were matched, the second peak decreased and disappeared, and further a positive signal was achieved. This behaviour confirmed the absorptiometric nature of this peak.

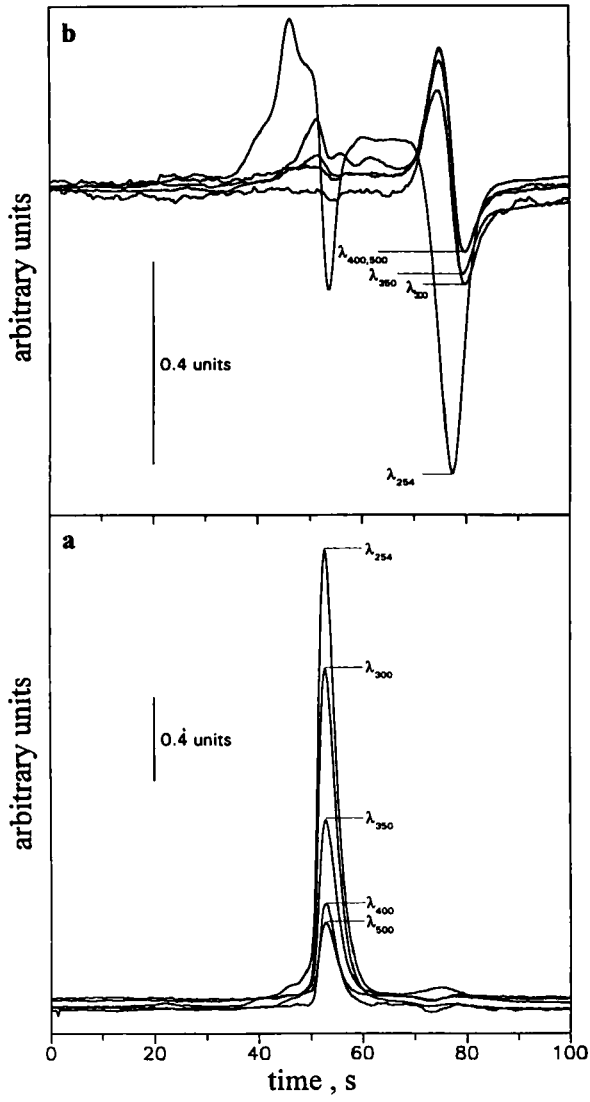

Figure 4. Influence of the wavelength in the measurement of the reference time obtained by injection of: (a) blank solution, and (b) water. A 0.05 M SDS mobile phase without modifier was used. Wavelengths are given in nm.

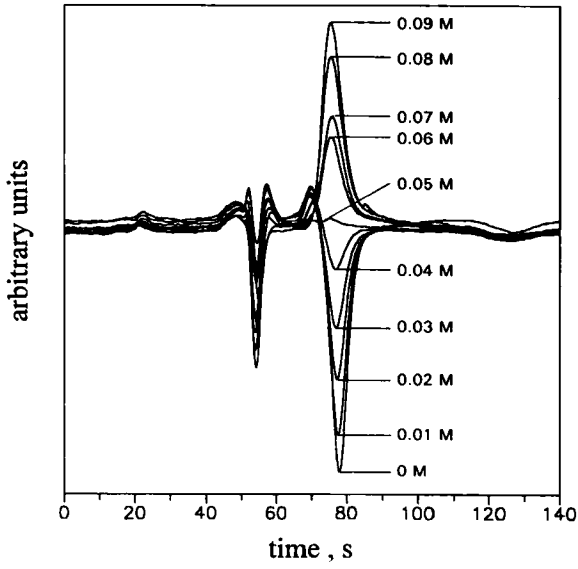

Figure 5. Influence of the concentration of SDS in the injected solution when a mobile phase of 0.05 M SDS without modifier was used

Figure 6 shows the head of four chromatograms corresponding to the injection of increasing volumes of ethacrynic acid dissolved in 0.05 M SDS into a mobile phase containing the same concentration of SDS. The start of the peak at 50 s was not modified, whereas its maximum was shifted to larger times for increasing volumes of the injected solution. This experience indicated that the measurement of a reference time is more convenient at the start of the perturbation than at its maximum.

The perturbation produced at the head of the chromatograms by injection of water, is 50 s wide, which suggested an extense diffusion of water in the mobile phase. It should be taken into account that the injected volume was 20 μL, and the flow-rate, 1 mL/min. If diffusion and retention do not take place, a narrow perturbation of about 1 s should be produced.

The values of reference time, retention time and capacity factor, k', for the injection of 14 replicates of a solution of amiloride into a mobile phase of 0.125 M SDS and 5% propanol are indicated in Table 1, according to each one of the four criteria proposed. The solution of amiloride was prepared in a medium with a composition similar to that of the mobile phase. The methods of groups led to the

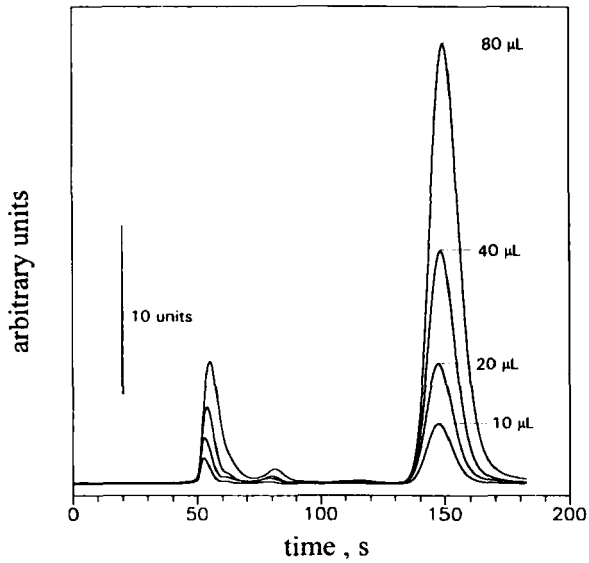

Figure 6. Influence of the volume of a solution containing 10 µg/mL of ethacrynic acid, injected into a mobile phase of 0.05 M SDS without modifier.

largest precision in the determination of the reference times and, consequently, in the calculation of capacity factors. The measurement at the maximum gave an acceptable precision, if the value of the sixth injection was eliminated ($t = 66.61 \pm 0.09$ s after rejecting the sixth injection). It should be considered that this series of experiences represents the most favorable case to obtain reproducible data, since successive injections of aliquots of the same solution were performed into the same mobile phase, in a short time period. It should be also noticed that replicate number 6 gave good results when the other three criteria were used. The obtention of such a discrepant measurement at the peak maximum suggested that this criterium is not very reliable.

Table 2 shows the values of reference time obtained in different mobile phases of SDS and butanol, when water, blank solution and diverse solutions of diuretics were injected. The blank solution and the solutions containing the diuretics had the same matrix and therefore, the head of their chromatograms should be similar. In effect, the mean values of the reference time obtained with both solutions did not show a significant difference. The standard deviations for both types of injected solutions are not comparable, since the number of replicates was different and

Table 1

Influence of the Reference Time on the Calculation of the Capacity Factor of Amiloride in a 0.125 M SDS/5% Propanol Mobile Phase

Replicate Number	Retention Time (s)	Reference Time(s)*				Capacity Factors **			
	t_r	t_1	t_2	t_3	t_4	k'_1	k'_2	k'_3	k'_4
1	370.20	66.76	60.37	59.76	60.77	4.54	5.13	5.19	5.09
2	358.82	66.55	60.56	59.73	63.00	4.39	4.92	5.01	4.70
3	369.90	66.72	60.51	59.88	61.20	4.54	5.11	5.18	5.04
4	368.94	66.50	60.53	59.84	59.94	4.55	5.10	5.17	5.16
5	369.36	66.69	60.48	59.79	60.60	4.54	5.11	5.18	5.10
6	369.00	60.64	60.48	59.84	61.00	5.08	5.10	5.17	5.05
7	368.94	66.59	60.45	59.65	60.37	4.54	5.10	5.18	5.11
8	369.00	66.49	60.49	59.75	60.60	4.55	5.10	5.18	5.09
9	369.06	66.54	60.49	59.89	60.60	4.55	5.10	5.16	5.09
10	368.94	66.57	60.60	59.82	60.34	4.54	5.09	5.17	5.11
11	368.82	66.57	60.50	59.80	60.30	4.54	5.10	5.17	5.12
12	370.14	66.66	60.46	59.84	60.32	4.55	5.12	5.19	5.14
13	369.48	66.72	60.46	59.81	63.00	4.54	5.11	5.18	4.86
14	368.82	66.67	60.46	59.72	60.12	4.53	5.10	5.18	5.13
Mean	368.5	66.2	60.49	59.79	60.9	4.57	5.09	5.16	5.06
St. dev.	2.8	1.6	0.05	0.07	1.0	0.15	0.05	0.05	0.13

* Reference time determined according to each criterium (1. first main maximum; 2. formation of groups scaling the noise; 3. formation of groups scaling the slope; 4. method of the first derivative).

** Capacity factors calculated using the reference time obtained according to each criterium.

reduced. Therefore, only the mean time values should be compared. However, the comparison of the standard deviations inside each criteria and for each type of injected solutions is interesting.

Table 2 also shows the maximum and minimum values of reference time obtained according to each criterium and with the eleven mobile phases studied. Measurement at the peak maximum led to very disperse values of time, not only

Table 2

Variation of the Reference Time with the Mobile Phase Composition

BuOH (v/v)	Criterium*	SDS (M) 0.050	0.075	0.100	0.125	0.150
				Reference time (s)		
0	1a	52.27-***	52.67±0.29	52.04±0.34**	52.10±0.14**	51.97±0.00**
	2a	51.60-***	49.30±0.00	51.46±0.08**	49.68±0.17**	49.89±0.44**
	3a	49.16-***	49.53±0.17	49.15±0.24**	49.66±0.14**	49.49±0.32**
	4a	49.17-***	49.61±0.10	49.42±0.07**	49.34±0.03**	49.38±0.03**
	1b	52.62-***	52.97±0.08	52.74±0.26**	52.68±0.05**	52.69±0.00**
	2b	49.53-***	52.31±0.16	52.40±0.00**	49.66±0.14**	49.63±0.03**
	3b	48.71-***	49.43±0.20	49.29±0.18**	49.58±0.47**	49.19±0.26**
	4b	49.67-***	49.82±0.12	49.86±0.14**	49.64±0.07**	49.91±0.10**
	1c	52.89±0.94	52.45±0.32	52.9±2.1	53.2±1.3	53.2±1.4
	2c	49.50±0.21	49.27±0.49	50.2±1.4	49.63±0.37	49.46±0.47
	3c	49.46±0.54	49.6±1.4	49.40±0.92	49.18±0.74	49.02±0.91
	4c	49.42±0.14	49.13±0.69	49.37±0.90	49.41±0.84	49.0±1.0
0.015	1a	50.20±0.10**		50.8±1.3**		49.60-***
	2a	50.33±0.15**		50.09±0.02**		49.91-***
	3a	50.67±0.11**		50.20±0.29**		50.06-***
	4a	50.48±0.06**		50.04±0.06**		50.18-***
	1b	53.22±0.15		52.96±0.09**		51.07±0.01**
	2b	49.80±0.08		50.27±0.71**		51.01±0.06**
	3b	50.05±0.06		50.06±0.04**		51.46±0.03**
	4b	50.04±0.02		49.94±0.02**		51.21±0.04**
	1c	53.26±0.10		53.18±0.31		52.3±1.3
	2c	49.86±0.12		50.09±0.16		50.68±0.39
	3c	50.06±0.07		50.07±0.18		51.30±0.63
	4b	49.96±0.13		49.96±0.18		50.70±0.49
0.030	1a	49.82±0.09**		50.9±1.5		48.74±0.64**
	2a	50.38±0.35**		50.04±0.03		50.21±0.21**
	3a	50.46±0.00**		50.30±0.16		50.38±0.21**
	4a	50.42±0.00**		50.06±0.10		50.32±0.12**
	1b	53.33±0.01		51.9±1.2**		54.63±0.07
	2b	49.76±0.01		49.94±0.05**		49.98±0.09
	3b	50.05±0.03		50.01±0.03**		50.30±0.02
	4c	50.06±0.02		49.93±0.00**		50.24±0.01

Table 2 (continued)

Variation of the Reference Time with the Mobile Phase Composition

BuOH (v/v)	Criterium*	SDS (M) 0.050	0.075	0.100	0.125	0.150
				Reference time (s)		
	1c	53.40±0.06		52.97±0.48		54.58±0.03
	2c	49.81±0.06		49.96±0.11		49.95±0.11
	3c	50.10±0.13		50.02±0.16		50.20±0.16
	4c	50.06±0.14		49.95±0.13		50.05±0.21

Mean Reference Time(s) in Blanks and Compound Solutions*

	t_1	t_2	t_3	t_4
Mean	53.07±0.62	49.96±0.38	49.85±0.64	49.79±0.47
Minimum	52.07	49.50	49.05	49.17
Maximum	54.57	50.75	51.33	50.80
Range	2.52	1.26	2.28	1.63

* Numbers 1 to 4 correspond to each mathematical criterium (see Table 1), and letters a, b, c indicate the solution that has been injected: water, blank and compound solution, respectively.
** Two measurements were available.
*** Only one measurement was available.

among the replicates done with different mobile phases, but also with the same mobile phase, the variation range being 2.52 s. On the other hand, the criterium of groups scaling the noise was the most precise, with a variation range of 1.26 s, which corresponds to an uncertainty of six experimental points in the chromatograms. The errors observed with the mobile phases containing 0.075 M and 0.1 M SDS were due to an anomalous peak start. The other two criteria, formation of groups scaling the slope and the method of the first derivative are similar, but the latter criterium gave somewhat better results.

The method of groups scaling the slope had a slight inertia with respect to the formation of groups scaling the noise, with delays of 0.2 to 0.3 s, for a flow-rate of 1 mL/min. This effect was due to the larger number of points used to calculate the slope. The inertia may be reduced by using a lower number of points, however the uncertainty in the calculation of the slope would be increased unacceptably. In spite of the slower response of the method of groups scaling the slope, this method may be preferable for very noisy chromatograms. Neither of the four criteria studied in this work was affected when the chromatograms showed an inclined base-line.

The numerous experiences performed with mobile phases of diverse composition, containing different concentrations of SDS and several alcohols, indicated that the reference time did no depend on the composition of the mobile phase. A small increase was only observed for the reference time determined at the maximum of the signal, which amounted to 0.5 and 2 s, when the concentrations of SDS or alcohol in the mobile phase were increased, from 0.05 to 0.15 M SDS, and from 0 to 3% butanol, respectively.

Figure 7 shows heads of chromatograms corresponding to the injection of salt solutions, KI and $NaNO_3$, and organic solvents, acetonitrile and methanol, and also of water and blank solution, into a 0.1 M SDS/12% propanol mobile phase. It may be observed that the shape of the perturbation is variable and more irregular with the injection of the salt solutions and organic solvents than with water or blank solution. Also, the perturbation began at a shorter time for the solutions of KI and $NaNO_3$. All the aqueous solutions injected gave a chromatogram with a maximum followed by a minimum around 68 s.

Influence of the Value Taken for the Dead Time on the Modelling of the Retention of Solutes

Equation (3) was used to study the influence of the value of the reference time in the prediction of capacity factors. The study was performed with the compounds: amiloride, bendroflumethiazide, chlorthalidone, spironolactone and triamterene. Modelling of the retention of these compounds was made with the retention data obtained in the eleven mobile phases indicated in Table 2. A non-linear fitting of the k' vs. ([M],) data to Equation (3), rewritten as:

$$k' = \frac{1 + A\varphi}{B\varphi + C[M] + D[M]\varphi + E} \qquad (7)$$

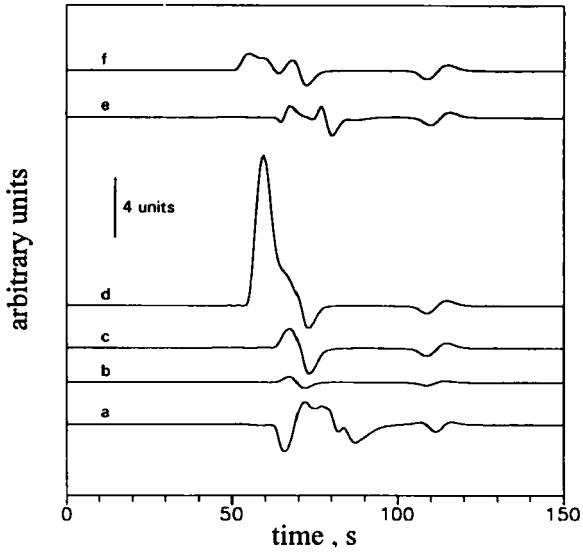

Figure 7. Heads of chromatograms obtained by injection of different solutions used to determine a reference time: (a) 10% acetonitrile/90% water; (b) blank micellar solution; (c) water; (d) potassium iodide; (e) methanol; and (f) sodium nitrate. Mobile phase: 0.1 M SDS/12% propanol. Manual injection was performed.

was made. This equation permits a more rapid convergence of the regression process. Next, the predicted and experimental capacity factors for each mobile phase were compared, and the mean relative error was calculated. Table 3 shows the errors of prediction of the capacity factors, obtained assuming different values of dead time. It is evident that the fitting of Equation (7) is scarcely affected by the errors introduced by using an incorrect dead time, at least up to a value of 80 s.

In contrast, Table 4 shows that the determination of the physico-chemical parameters K_{SW}, K_{AM}, K_{AD}, K_{MD} and K_{SD} is severely affected by the value taken for the dead time, especially for the three former constants. Changes of sign in these parameters are often observed, which indicated that the dead time used in the calculation is not correct. Large errors in the dead time can lead to ill-conditioned fittings that give non-sense negative parameters. The significant changes in the values of the parameters for the five compounds shown in Table 4, that occur between 40 and 60 s suggested that the real dead time (including the residence time in pre- and post-column conducts) belongs to this time range. Similar results were obtained for mobile phases without modifier, the physico-chemical parameters K_{SW} and K_{AM} being sensitive to the value of dead time.

Table 3

Influence of the Value of Dead Time in the Prediction of Capacity Factors

Mean Relative Error %

Dead Time(s)	Amiloride	Bendroflu-methiazide	Chlorthali-done	Spirono-lactone	Triamterene
30	1.19	1.14	1.14	1.27	1.29
40	1.19	1.08	1.11	1.19	1.20
50	1.21	1.05	1.08	1.10	1.11
60	1.23	1.06	1.06	1.02	1.02
70	1.28	1.13	1.05	0.93	0.93
80	1.44	1.37	1.26	0.83	0.84
90	1.62	1.71	1.79	0.73	0.74
110	2.02	3.00	4.35	0.77	0.64
130	2.55	5.32	12.99	0.96	0.81

CONCLUSIONS

The determination of the real dead time is not an easy task when a micellar mobile phase is used. The observation of a large number of chromatograms showed that the shape, height and sign of the first peak is unpredictable, especially when the nature of the injected solution is different to that of the mobile phase. It was confirmed that the usual practice of taking the time measured at the maximum of the first peak, as the value of dead time, is not correct. The time measured should only be considered as a reference time. The application of the four criteria proposed in this work to the study of the first perturbation gave different values of reference time for each type of injected solution (water, blank, salt solutions and organic solvents).

In the literature, the dead time is usually determined by injection of water. However, this procedure is not very reproducible, especially when the time is measured at the maximum of the first peak, owing to the large difference in composition between water and the micellar mobile phase. The measurement of the reference time at the peak maximum is very simple, but the variability in the shape and position of the first peak leads frequently to unprecise values. In contrast, the start of the first main peak in the chromatograms is fairly reproducible and probably close to the dead time. Among the methods proposed to locate this point, the method of groups scaling the noise gave the best results.

Table 4

Influence of the Value of Dead In the Determination of Physico-Chemical Parameters

Compound	Dead Time(s)	R	Physico-Chemical Parameters				
			K_{SW}	K_{AM}	K_{AD}	K_{MD}	K_{SD}
Amiloride	30	0.99992	1040	377	8.32	1.33	0.481
	40	0.99992	988	487	9.62	1.35	0.473
	50	0.99993	1092	685	11.91	1.38	0.465
	60	0.99993	1502	1152	17.23	1.41	0.457
	70	0.99993	3934	3588	44.66	1.45	0.448
	80	0.99992	-3050	-3241	-31.96	1.48	0.440
	90	0.99992	-919	-1120	-8.05	1.52	0.431
	110	0.99990	-314	-487	-0.75	1.60	0.413
	130	0.99986	-164	-312	1.43	1.69	0.394
Bendroflu-methiazide	30	0.99990	121	91	1.97	0.36	0.153
	40	0.99992	102	107	2.11	0.37	0.146
	50	0.99994	95	129	2.31	0.39	0.138
	60	0.99995	95	163	2.58	0.41	0.130
	70	0.99995	105	220	3.02	0.44	0.121
	80	0.99994	134	336	3.86	0.46	0.113
	90	0.99992	237	704	6.43	0.49	0.103
	110	0.99981	-152	-611	-2.38	0.56	0.083
	130	0.99958	-41	-216	0.50	0.66	0.061
Chlorthali-done	30	0.99986	54	48	1.74	0.14	0.038
	40	0.99989	44	55	1.85	0.16	0.026
	50	0.99992	38	63	1.97	0.18	0.013
	60	0.99994	35	75	2.12	0.21	-0.001
	70	0.99995	35	92	2.31	0.24	-0.015
	80	0.99995	37	119	2.58	0.28	-0.031
	90	0.99992	43	165	2.99	0.32	-0.047
	110	0.99976	131	715	6.66	0.44	-0.084
	130	0.99930	-43	-321	0.86	0.64	-0.124

(continued)

Table 4 (continued)

Influence of the Value Of Dead in the Determination of Physico-Chemical Parameters

Compound	Dead Time(S)	R	Physico-Chemical Parameters				
			K_{SW}	K_{AM}	K_{AD}	K_{MD}	K_{SD}
Spirono lactone	30	0.99997	2832	570	15.72	1.33	0.301
	40	0.99998	3864	1053	27.45	1.34	0.297
	50	0.99998	18416	6367	156.30	1.36	0.293
	60	0.99998	-3638	-1532	-35.26	1.38	0.289
	70	0.99999	-1387	-692	-14.83	1.40	0.285
	80	0.99999	-769	-445	-8.82	1.42	0.280
	90	0.99999	-496	-328	-5.97	1.44	0.276
	110	0.99999	-257	-215	-3.18	1.49	0.268
	130	0.99999	-157	-160	-180	1.54	0.259
Triamterene	30	0.99995	300	610	16.63	1.30	0.298
	40	0.99995	2772	759	19.43	1.33	0.292
	50	0.99996	2903	1003	24.00	1.35	0.287
	60	0.99996	3529	1478	32.82	1.37	0.281
	70	0.99997	5679	2805	57.31	1.40	0.275
	80	0.99997	46948	26778	498.73	1.42	0.269
	90	0.99997	-5488	-3559	-59.70	1.45	0.263
	110	0.99998	-1349	-1092	-14.13	1.50	0.251
	130	0.99998	-662	-646	-5.75	1.57	0.238

The reference time is better measured by injecting the blank solution and the micellar solutions of the solutes, especially if the detection is performed at a low wavelength, in order to remark the differences in composition between the injected solution and mobile phase. An additional advantage is the large number of individual values of the reference time that can be easily achieved, since any injection of solutes may be used to measure the reference time. The determination of the reference time in a large number of different mobile phases showed that it did not change appreciably with the composition of the eluent. This suggested that the same value of reference time can be used to predict capacity factors of solutes eluted in a given chromatographic column, with mobile phases containing variable amounts of surfactant and alcohol.

In experimental design and in the search of the optimum mobile phase to separate a mixture of solutes, the use of an approximate value of the dead time leads to good results, since the capacity factors are only used as intermediate values in the prediction of the position of the chromatographic peaks, expressed as retention time. However, in the calculation of capacity factors, the same value of dead time must be used for all mobile phases. On the other hand, the evaluation of physico-chemical retention parameters requires the use of an accurate value of dead time. The methods that search the start of the main first perturbation on the chromatograms give satisfactory constants.

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