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Combined pH/Organic Modifier Double Gradient Reversed-Phase HPLC*

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A new procedure of a combined pH and organic solvent double gradient reversed-phase high-performance liquid chromatography (RP HPLC) has been proposed. The procedure is technically feasible and theoretically proved. The method is demonstrated to outstandingly enhance separation of ionizable analytes. The approach consists in simultaneous developing of linear gradients of pH and of the organic modifier in the mobile phase. An extraordinary, systematically increasing eluting power of the mobile phase leads to a remarkable analyte peak compression, accompanied by a strongly reduced peak tailing.

Key words: pH gradient/organic gradient HPLC, pK_a, ionizable analytes

Since some time gradient mode reversed-phase high performance liquid chromatography (RP HPLC) has routinely been used for separations of analytes, particularly those which are difficult to separate with the standard isocratic mode. By the term ,,gradient RP HPLC" one normally understands the programmed change (usually linear) of the eluting power of the mobile phase during the chromatographic run. That change (an increase) is provided by adding a stronger (organic) solvent B, *e.g.*, methanol or acetonitrile, to a weaker solvent A, usually water. Such organic-solvent-gradient in RP HPLC has been described in theoretical terms [1–4] and is more and more often used in chemical and biomedical analysis [5].

Retention of ionogenic analytes in reversed-phase HPLC is known to strongly depend on the pH of the eluent. Retention factor, k, of a nondissociated form of an acid or a base may be 10–20 times larger than that of the respective dissociated form at given composition of the water-organic mobile phase. A theory applicable to isocratic systems was first presented and tested for a buffered water mobile phase by Horvath *et al.* [6]. Latter on Van de Venne *et al.* [7] extended the studies on the relationships between eluent pH and analyte retention to the methanol-water mobile phases. A full theoretical model of retention in isocratic RP HPLC as a function of pH at fixed eluent compositions was proposed by Lopes Marques and Schoenmakers [8].

Up to now, the literature reports [9–13] on the use of pH modifications to affect HPLC separations have referred to the pH value which is kept constant during total

* Dedicated to Prof. E. Borowski on the occasion of his 75th birthday.

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chromatographic run. We found it technically feasible to carry out reversed-phase HPLC with linearly changing pH of the water (buffer)-organic eluents of a fixed organic solvent content [14–16]. Such pH gradient RP HPLC is done by linearly decreasing (in case of base analytes) or increasing (in case of acids) the pH of the eluent [17], thus providing a functional increase in analyte dissociation and consequently, a decrease in its retention. The pH gradient RP HPLC can be of particular bioanalytical importance because several compounds undergo structural and functional changes at higher concentrations of organic solvents, whereas most such compounds tolerate well the changes in pH.

Having successfully implemented the pH gradient RP HPLC [14–17], we came to conclusion that a combination of the two gradients, pH and organic solvent, could make sense. Here, we report the theoretical background of the new RP HPLC procedure and present exemplary results.

In chromatography, the fundamental equation describing analyte retention, valid for either the organic modifier gradient or the pH gradient, is [18,19]:

$$dx = \frac{dV}{V_0 k_i} \tag{1}$$

where V denotes the volume of mobile phase passing through the inlet of the column since the start of gradient, V_0 is the void ("dead") volume (*i.e.,* retention volume of a nonretained marker), k_i is the retention factor of the analyte at the beginning of the column and dx is a fractional analyte band migration through the column. Obviously, the equation also holds in isocratic mode, where k_i is constant. For a unit column $\Sigma dx = 1$ and the retention volume parameters can be replaced with the corresponding retention time parameters. Thus, one gets:

$$\int_0^{t_R'} \frac{1}{t_0}\frac{dt}{k_i} = 1 \tag{2}$$

where $t_R' = t_R - t_0$ denotes the reduced retention time, *i.e.*, the measured gradient retention time, t_R, less the void time, t_0. In the combined pH/organic solvent gradient RP HPLC both pH and the content, φ, of organic modifier in eluent change with time. With proper buffers these changes can be programmed linear:

$$pH = pH_0 + a(t - t_d) \tag{3}$$

$$\varphi = \varphi_0 + b(t - t_d) \tag{4}$$

where subscript zero means the starting values, t_d is the chromatographic system's dwell time, whereas a and b are the respective gradient steepness parameters.

Assuming that added amounts of organic modifier (here: methanol) do not change markedly the pH of the eluent [8,10], the k_i in Eq. 2 changes during the pH gradient elution as follows (in case of base analytes):

$$k_i = \frac{k_{[BH^+]} + k_{[B]} 10^{pH(t) - pK_a}}{1 + 10^{pH(t) - pK_a}} \tag{5}$$

In the combined pH/organic solvent gradient RP HPLC, the changing content of organic modifier affects the retention of both the ionized, BH^+, and the nonionized, B, form of base analytes. These changes are described by the well known equations [1]:

$$\log k_{BH^+} = \log k_{wBH^+} - S_{BH^+}\varphi(t) \tag{6}$$

$$\log k_B = \log k_{wB} - S_B\varphi(t) \tag{7}$$

where subscript w accounts for the retention factor in a (hypothetical) neat water eluent; S is a constant, which is characteristic for the analyte and the chromatographic system. Assuming that the changing content of methanol does not alter dramatically the pK_a of the analyte and putting Eqs. 5, 6 and 7 into Eq. 2, one gets:

$$\int_0^{t_R'} \frac{1}{t_0} \frac{1 + 10^{pH(t) - pK_a}}{10^{\log k_{wBH^+} - S_{BH^+}\varphi(t)} + 10^{\log k_{wB} - S_B\varphi(t)} 10^{pH(t) - pK_a}} dt = 1 \tag{8a}$$

or:

$$\int_0^{t_R'} \frac{1}{t_0} \frac{1 + 10^{pH(t) - pK_a}}{k_{wBH^+} 10^{-S_{BH^+}\varphi(t)} + k_{wB} 10^{pH(t) - pK_a - S_B\varphi(t)}} dt = 1 \tag{8b}$$

Equations 8a and 8b are the general equations describing retention in any chromatographic mode, whether pH and/or organic solvent content change or not, *i.e.*, in isocratic, organic solvent gradient, pH gradient and the combined pH/organic solvent gradient models.

Having Eqs. 3 and 4 and assuming that up to the dwell time, t_d, the retention is negligible, for the analytes eluted at $t_R < t_0 + t_d + t_G$, where t_G is duration of gradient, one arrives at Eq. 9.

$$\int_{t_D}^{t_R'} \frac{1}{t_0} \frac{1 + 10^{pH_0 + a(t - t_d) - pK_a}}{k_{wBH^+} 10^{-S_{BH^+}[\varphi_0 + b(t - t_d)]} + k_{wB} 10^{pH_0 + a(t - t_d) - pK_a - S_B[\varphi_0 + b(t - t_d)]}} dt = 1 \tag{9}$$

When the duration of pH gradient differs from the duration of the organic modifier gradient in the combined pH/organic solvent double gradient RP HPLC, the total time of gradient duration can be divided into stages. For example, one can consider a two-stage gradient, *i.e.*, when organic gradient is developed linearly from, *e.g.*, $\varphi_0 = 0.06$ to $\varphi_2 = 0.50$ at time from $t_0 = 0$ min to $t_2 = 20$ min and the pH changes linearly from, *e.g.*, 11.50 to 2.50 within the first 10 min. After $t_1 = 10$ min φ_1 attains the value 0.28.

When the analyte is eluted at $t_d < t'_R < t_1 + t_d$, the following equation describes the retention (assuming that dwell time is zero or the retention during dwell time is negligible):

$$\int_{t_d}^{t'_R} \frac{1}{t_0} \frac{1 + 10^{pH_0 + a_1(t - t_d) - pK_a}}{k_{wBH^+} 10^{-S_{BH^+}[\varphi_0 + b_1(t - t_d)]} + k_{wB} 10^{pH_0 + a_1(t - t_d) - pK_a - S_B[\varphi_0 + b_1(t - t_d)]}} dt = 1 \quad (10)$$

When analyte is eluted at $t_1 + t_d < t'_R < t_2 + t_d$, the retention equation is a sum of two parts:

$$\int_{t_d}^{t_1 + t_d} \frac{1}{t_0} \frac{1 + 10^{pH_0 + a_1(t - t_d) - pK_a}}{k_{wBH^+} 10^{-S_{BH^+}[\varphi_0 + b_1(t - t_d)]} + k_{wB} 10^{pH_0 + a_1(t - t_d) - pK_a - S_B[\varphi_0 + b_1(t - t_d)]}} dt + \int_{t_2 + t_d}^{t'_R} \frac{1}{t_0} \frac{1 + 10^{pH_1 + a_2(t - t_d) - pK_a}}{k_{wBH^+} 10^{-S_{BH^+}[\varphi_1 + b_2(t - t_d)]} + k_{wB} 10^{pH_1 + a_2(t - t_d) - S_B[\varphi_1 + b_2(t - t_d)]}} dt = 1 \quad (11)$$

where a_1, a_2 denote the steepness of pH gradient developed for individual stages and b_1, b_2 denote the slope of the organic solvent gradient at the corresponding stage. A suitable equation can be found for even more complex situations by analogy.

EXPERIMENTAL

Chromatographic measurements were done using a Merck-Hitachi LaChrome (Darmstadt, Germany-San Jose, CA, USA) apparatus equipped with a diode array detector, autosampler and thermostat. The measured value of the dwell volume, V_d, was 1.4 ml. Chromatographic data were collected using D-7000 HPLC System Manager, version 3.1 (Merck-Hitachi). Numerical analysis and data processing were done with Matlab® Software version 6.5.0 (The MathWorks, Inc., Natick, MA, USA).

The column used was XTerra MS C-18, 150×4.6 mm I.D., particle size 5 μm (Waters Corporation, Milford, MA, USA). Mobile phases contained methanol as the organic modifier (solvent B). Water or buffers (I and II) of a fixed pH formed the aqueous component of the eluent (solvent A). Buffers of ${}^{w}_{w}pH$= 3.00 (buffer I) and ${}^{w}_{w}pH$= 10.50 (buffer II), mixed at various proportions, formed the solvent A. The ${}^{w}_{w}pH$ of the buffers was measured at 25°C. The measurements were done with an HI 9017 pH-meter (Hanna Instruments, Bedfordshire, UK). Essential buffer solution formed three compounds, each at the concentration of 0.004 M: citric acid (CIT), tris(hydroxymethyl)aminomethane (TRIS), 3-(cyclohexylo-amino)-1-propane sulfonic acid (CAPS). Buffer I was made by adding to the essential solution 1 M HCl to obtain the desired pH. Buffer II was made by adding necessary amounts 1 M NaOH. During the pH gradient run buffers II and I were mixed in a mix chamber together (forming solvent A) with a fixed content of methanol (solvent B). The composition of buffer was established in preliminary experiments such as to provide the linear change of pH during the pH gradient developed with a fixed methanol content in the mobile phase. 1% urea was used to determine the column dead volume, V_o. Such determined V_o was 1.72 ± 0.02 ml. RP HPLC measurements were done at 35°C with eluent flow-rate of 1.0 ml/min. All the reagents and analytes employed were of a highest commercially available quality.

RESULTS AND DISSCUSION

Figure 1 presents the results of modeling of retention by Eq. 9 for a hypothetical analyte in various HPLC modes. The chromatographic conditions applied are described in Table 1. In the figure the sum of fractional analyte migration is plotted against time and the theoretically calculated reduced retention time, t'_R, is indicated at the top of figure. Calculations by numerical integration consisted in finding a function, *y(t)*, whose first derivative, *y'(t)*, is the integrand in Eq. 9. Function *y(t)* is a sum of fractional migrations in time. When $y(t) = 1$ then, according to Eq. 9, the elution of the analyte from the column takes place at reduced retention time. For a hypothetical analyte in Fig. 1, a typical characteristic was assumed: $k_{wB} = 100$, $k_{wBH^+} = 10$, $S_B = S_{BH^+} = 3.5$ and $pK_a = 9$. The parameters of the HPLC system, $t_0 = 1$ min and $t_d = 0$ min, were assumed for the calculations.

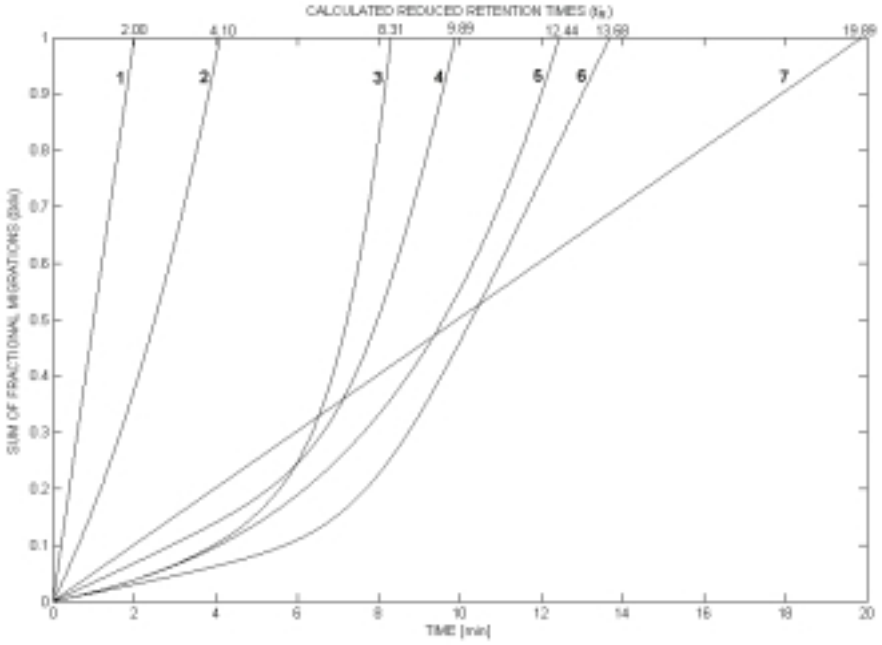


Figure 1. Fractional migration of a hypothetical analyte as a function of time. Calculations were done by Eq. 9 assuming the parameters of the analyte and of the chromatographic system as specified in the text. The plot numbers refer to the following chromatographic conditions: 1 – isocratic mode, ionized analyte, pH 2.50, φ 0.20; 2 – organic solvent gradient, ionized analyte, pH 2.50, φ 0.05–0.60; 3 – combined pH/organic solvent double gradient, pH 11.50–2.50, φ 0.05–0.60; 4 – pH gradient (conditions I), pH 11.50–2.50, φ 0.05; 5 – organic solvent gradient nonionized analyte, pH 11.50, φ 0.05–0.60; 6 – pH gradient (conditions II), pH 11.50–2.50, φ 0.15; 7 – isocratic, nonionized analyte, pH 11.50, φ 0.20.

Table 1. Conditions of individual RP HPLC modes.

No.	Mode (Analyte Form in Parentheses)	RP HPLC Conditions
1	Organic solvent gradient (Ionized)	pH 2.50, φ 0.05–0.60, t_G 20 min
2	Combined pH/organic solvent gradient	pH 11.50–2.50, t_{G1} 10 min; φ 0.05–0.60, t_{G2} 20 min
3	Combined pH/organic solvent gradient	pH 11.50–2.50, φ 0.20–0.60, t_G 20 min
4	Combined pH/organic solvent gradient	pH 11.50–2.50, φ 0.05–0.60, t_G 20 min
5	pH gradient I	pH 11.50–2.50, φ 0.25, t_G 20 min
6	pH gradient II	pH 11.50–2.50, φ 0.15, t_G 20 min
7	pH gradient III	pH 11.50–2.50, φ 0.05, t_G 20 min
8	Organic solvent gradient (Nonionized)	pH 11.50, φ 0.05–0.60, t_G 20 min

In combined pH/organic solvent double gradient run (curve 3 in Fig. 1) the steepness of the plot of the distance passed by the analyte within the column against time increases as compared to the organic gradient alone (curve 5, Fig. 1). That increase start since the pH has decreased enough to induce analyte dissociation.

As a matter of fact, elution under organic solvent gradient alone at pH providing a full analyte dissociation (curve 2 in Fig. 1) might be even faster. The quality of RP HPLC separations of dissociated analytes, especially bases, would be poor, however.

The presumed advantages of the combined pH/organic solvent double gradient RP HPLC were tested experimentally. In Fig. 2 exemplary chromatograms of a mixture of 14 low molecular mass basic analytes are given as obtained at comparable conditions (Table 1). In case of combined pH/methanol gradient, the peak height of 2,4,6-collidine is 6-fold larger than that obtained with single methanol gradient. Retention times, peak signal intensity and theoretical plate number are given in Table 2.

The combined pH/methanol double gradient developed for 25 min (Fig. 2a) produced a complete separation of the mixture. Methanol gradient of 25 min at pH stopping the dissociation of the analytes (pH = 11.50) did not separate some analytes, like codeine from brucine and N-ethylaniline from 2,4,6-collidine (Fig. 2b). Also, the intensity of peak signals and the corresponding numbers of theoretical plates are for the most retained peaks several times worse than in the case of the combined pH/methanol double gradient mode. The pH gradient alone (Fig. 2c) gives separation of the test mixture but requires longer time. On the other hand, the isocratically obtained separation at pH = 11.50, suppressing the ionization of analytes, is poor and needs twice as long time (Fig. 2d).

One can note in Fig. 2a-c that all the gradients modes provide intense, narrow, symmetrical peaks with minimized tailing. In Fig. 2a, the later eluting peaks are narrower than the first ones, which is not the case with the single gradients (Figs. 2b and 2c). The explanation is that in the combined pH/organic double gradient RP HPLC (Fig. 2a) the first peaks are affected by the organic solvent gradient only. It is so because the first analytes are eluted from the column before the pH of eluent decreases enough to provide analytes dissociation and to accelerate their migration.

Table 2. Characteristics of individual chromatograms of Fig. 2. Theoretical plate number, *N*, was calculated as $N = 2\pi\left(\frac{t_R h}{A}\right)^2$, where h is a peak signal measured by a UV detector and A is the area of the peak.

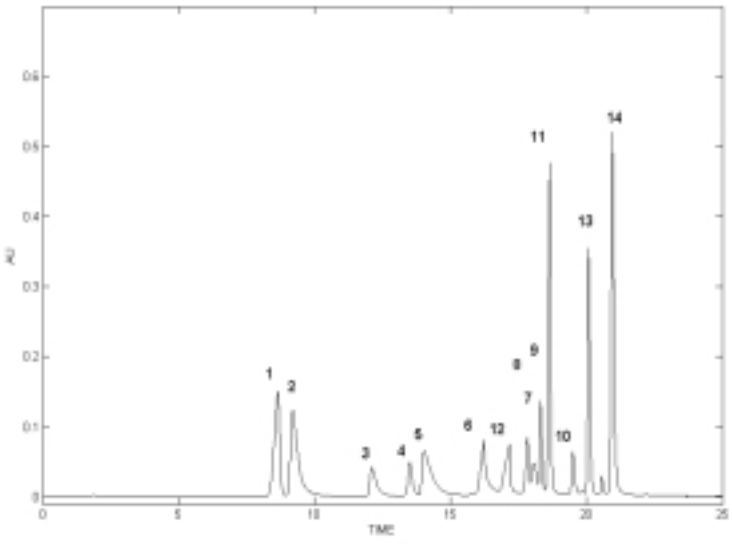
No.	Analyte	Chromatogram A			Chromatogram B			Chromatogram C			Chromatogram D		
		Combined pH/Organic Solvent Gradient			Organic Solvent Gradient (pH 11.50)			pH Gradient			Isocratic 30 % v/v MeOH (pH 11.50)		
		t_R [min]	h [AU]	N×10^3	t_R [min]	h [AU]	N×10^3	t_R [min]	h [AU]	N×10^3	t_R [min]	h [AU]	N×10^3
1	Morphine	8.650	0.149	8.26	7.867	0.152	15.78	2.417	0.118	0.25	2.667	0.082	0.54
2	Aniline	9.200	0.123	4.69	9.333	0.133	12.86	3.867	0.205	1.52	4.133	0.149	2.89
3	2-Amino-5-nitropyridine	12.117	0.041	9.83	11.183	0.047	24.11	4.517	0.054	1.33	4.817	0.039	2.61
4	2-Methylbenzimidazole	13.517	0.048	27.57	12.217	0.044	48.22	5.450	0.040	2.06	5.950	0.026	4.05
5	Benzylamine	14.033	0.075	6.23	12.567	0.086	24.85	6.100	0.096	1.80	6.617	0.057	3.11
6	N-Methylaniline	16.217	0.080	22.45	14.200	0.061	43.83	8.700	0.038	2.69	9.700	0.026	4.45
7	2,2′-Bipyridene	18.050	0.046	46.47	14.850	0.037	73.05	10.600	0.019	2.73	11.783	0.012	4.70
8	Codeine	17.833	0.083	106.37	16.500[3)]	0.118[3)]	65.70[3)]	18.000	0.075	69.29	29.533	0.008	8.34
9	Brucine	18.317	0.135	161.86				19.633	0.108	84.17	45.650	0.005	6.99
10	N-Ethylaniline	19.500	0.062	128.53	16.900[3)]	0.166[3)]	77.03[3)]	15.933	0.027	4.89	18.117	0.016	5.38
11	2,4,6-Collidine	18.650	0.475	164.57				18.717	0.225	30.52	24.083	0.024	7.24
12	N-Benzyldimethylamine	17.167	0.073	24.84	17.767[3)]	0.045[3)]	104.66[3)]	16.867	0.075	16.93	32.450	0.005	5.39
13	Papaverine	20.083	0.352	220.21	18.033	0.044	93.05	26.650	0.169	48.27	96.452[1)]	[2)]	[2)]
14	Acridine	20.967	0.519	167.23	19.300	0.144	121.62	25.383	0.468	111.71	105.567	0.008	9.54

[1)] Determined in a separate experiment; [2)] not measurable; [3)] analytes were not separated.

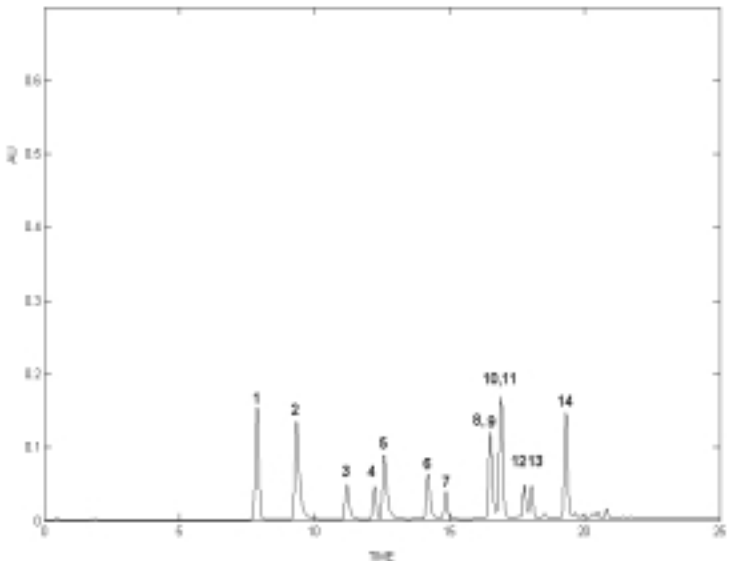
On the other hand, compounds with high log k_w and pK_a, *e.g.*, N-benzyldimethylamine, $pK_a = 8.91$, are accelerated by pH gradient already at the beginning of column. In consequence, such analytes (actually, their ionized forms) are also affected by the organic solvent gradient.

A combined pH/organic double gradient technique may be realized with a standard HPLC equipment. It offers a simple means to significantly improve the quality of RP HPLC separations.

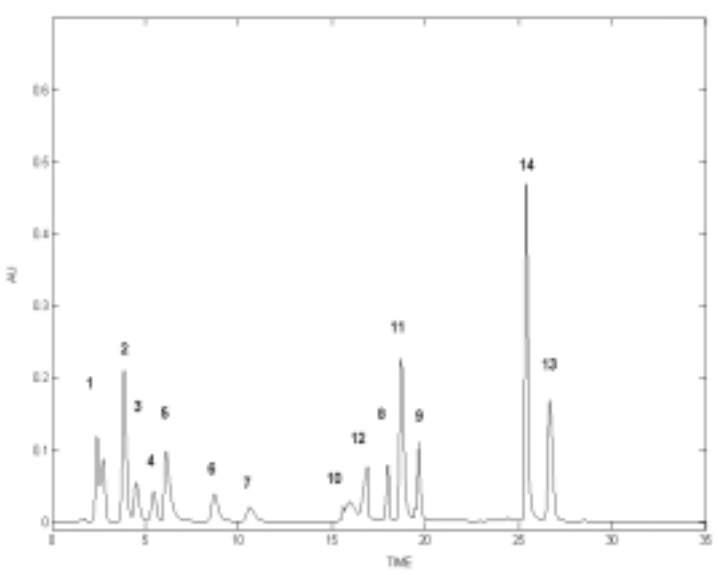
a)



b)



c)



d)

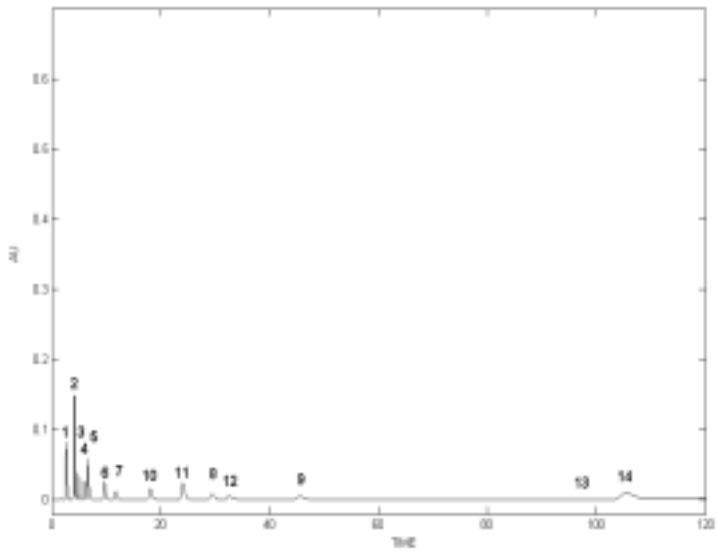


Figure 2. Chromatograms of a test mixture of analytes obtained at the following conditions: a) combined pH/methanol gradient; pH from 11.50 to 2.50; φ from 0.05 to 0.50; t_G 15 min; b) methanol gradient at pH 11.50; φ from 0.05 to 1.00; t_G 20 min; c) pH gradient; pH from 11.50 to 2.50; φ 0.30; t_G 20 min; d) isocratic condition; φ 0.30; pH 11.50. The same scale on ordinate axis is on all the chromatograms. Analyte numbers correspond to: 1 – morphine, 2 – aniline, 3 – 2-amino-5-nitropyridine, 4 – 2-methylbenzimidazole, 5 – benzylamine, 6 – N-methylaniline, 7 – 2,2′-bipyridene, 8 – codeine, 9 – brucine, 10 – N-ethylaniline, 11 – 2,4,6-collidine, 12 – N-benzyldimethylamine, 13 – papaverine, 14 – acridine.

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