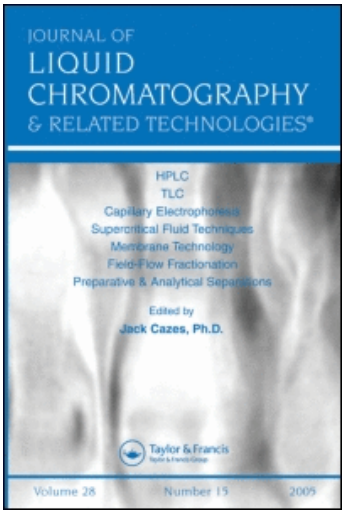

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Study and Optimization of Column Efficiency in HPLC: Comparison of Two Methods for Separating Ten Benzodiazepines

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STUDY AND OPTIMIZATION OF COLUMN EFFICIENCY IN HPLC: COMPARISON OF TWO METHODS FOR SEPARATING TEN BENZODIAZEPINES

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ABSTRACT

To understand the influence of mobile phase composition, its flow rate and column temperature involved in high performance Liquid Chromatography, an experimental design was used. The observed responses were the theoretical plate number, the linear velocity of the mobile phase and a new chromatographic resolution function which provided the most efficient separation of ten compounds as ten benzodiazepines. Optimum conditions obtained were compared with another optimization method.

INTRODUCTION

The column efficiency in high performance liquid chromatography (HPLC) has been widely studied and is generally represented by the height equivalent to a theoretical plate (HETP). HETP was first related to the mobile phase flow rate by Knox (1). To optimize the column efficiency, the traditional approach would be to study separately the mobile phase composition, its flow rate and column temperature which influences HETP (2). In this research, an experimental design (3, 4), assisted with a simplex method was used to study the simultaneous variation of the mobile phase composition, its flow rate and column temperature. An equation relating the column efficiency with these three factors was proposed. A new chromatographic

resolution function (CRF) (5, 6, 7, 8) was studied for the separation of several compounds and this method was compared with that developed in a recent work (9).

MATERIALS AND METHODS

CHROMATOGRAPHIC CONDITIONS

APPARATUS : The HPLC system consisted of a HPLC Waters pump 501 (Saint Quentin en Yvelines, France), an Interchim rheodyne injection valve Model 7125 (Montluçon, France) fitted with a 20µl sample loop, a Merck L 4000 variable wavelength UV spectrophotometer detector and a Merck D 2500 chromato integrator (Nogent-sur-Marne, France). A Waters 150 mm × 3.9 mm ID. RP 18 column (Nova pak, 5 µm particle size) was used with a controlled temperature by an Interchim crococil oven TM N° 701 (Montluçon, France). Overall temperature control was maintained within ± 1° C with a variation from 26° C to 50° C. The detection wavelength was 254 nm. The flow rate used varied from 0.6 to 1.6 mL/min. The mobile phase was a mixture methanol-water with varied percentages of methanol from 50 % to 80 %. Weaker percentages were not used because of the excessively high column pressure obtained with 50 % of methanol with a flow rate of 1.6 mL/min.

REAGENTS AND SAMPLES : Methanol was HPLC grade determine analytical. Naphtalene obtained from Merck (Nogent-sur-Marne, France) was used to determine the theoretical plate number (N). The linear velocity (u) was measured by timing the passage of an unretained peak such as sodium nitrate purchased from Merck (Nogent-sur-Marne, France). (1) Bromazepam (2) Nitrazepam (3) Flunitrazepam (4) Clobazam (5) Lorazepam (6) Oxazepam (7) Tofisopam (8) Chlordiazepoxide (9) Chlorazepate dipotassic and (10) Diazepam were obtained from HOFFMANN LA ROCHE (Basel, Switzerland). These were diluted in methanol in a concentration range of 10-80 mg/mL.

METHODS

EFFICIENCY : In chromatography, the column plate number (N) is used to study the sharpness of a peak. N depends on the mobile phase composition, its flow rate and column temperature. N is given by the following equation :

Table 1. Experiments required for a three variable experimental design

Experiments N	Methanol (% v/v)	Flow rate (mL/min)	Temperature (C)
1	50	0.6	36
2	50	1.0	50
3	50	1.6	36
4	50	1.0	26
5	63	0.6	26
6	63	0.6	50
7	63	1.6	50
8	63	1.6	26
9	80	1.0	50
10	80	0.6	36
11	80	1.0	26
12	80	1.6	36
13	63	1.0	36

$$N = \left(\frac{t_R}{s}\right)^2 \qquad [1]$$

where t_R is the retention time of naphtalene, s its standard deviation considering the peak as gaussian. N is calculated using the peak width ($W_{0.5}$) at half height using :

$$N = 5.54\left(\frac{t_R}{W_{0.5}}\right)^2 \qquad [2]$$

The theoretical plate number is directly proportional to the column length (L). Thus using : the height equivalent to a theoretical plate HETP can be calculated:

$$HETP = \frac{L}{N} = \frac{L}{5.54}\left(\frac{W_{0.5}}{t_R}\right)^2 \qquad [3]$$

LINEAR VELOCITY : A fundamental parameter affecting the separation speed and the column plate number is the linear mobile phase velocity (u) in millimeters per second. It was measured by timing the passage of an unretained peak such as sodium nitrate (t_m) along the length of the column L.

$$u = \frac{L}{t_m} \qquad [4]$$

CHROMATOGRAPHIC RESOLUTION FUNCTION : The quality of each separation of ten compounds was assessed at the end of the chromatogram by calculating the value of a chromatographic resolution function CRF which describes the separation quality. CRF is usually given by the following equation :

$CRF = \alpha F_{obj} + \beta m^a$ [5]

where F_{obj} = objective function

m = detected peak number

α, β and a are constants.

F_{obj} is expressed in terms of resolution Rsij between two peaks i and j (10, 11, 12). But Rsij is insensitive to the relative quantity of solutes in the mixture. For a badly separated pair of compounds injected into a diluted solution, the difference between the solute quantity which is represented by the amplitude difference between peaks leads to an error in the compound quantification particularly for the minority compounds. Although the resolution is constant, this error increases drastically when the amplitude ratio is high. Some authors have tried to find another separation function which takes into account the amplitude ratio of peaks by a direct measurement on the chromatogram : Kaiser ratio (13) Christophe ratio (14). More recently EL Fallah and Martin (15) have introduced a discrimination factor $d_0 = \frac{h_p - h_v}{h_{..}}$ where h_p - h_v is the highest difference between the smallest peak (h_p) and the valley (h_v) separating the two peaks. In this paper, a new chromatographic resolution function was studied where Fobj is a function of d_0 given by the following equation

$F_{obj} = \sum \ln (1 + d_{ij})$ [6]

where d_{ij} is the discrimination factor between peak i and j. The sum is extended to all the peak pairs on the chromatogram.

CHEMOMETRIC METHODOLOGY : A chemometric approach based on the use of Box and Benhken matrix experiments (3) [Fig.1] was used to study simultaneously the variations in all the factors. These models can be used for regression analysis and for three factors takes the form of:

$y = a_0 + a_1 \ln x_1 + a_2 \ln x_2 + a_3 \ln x_3 + a_{11}(\ln x_1)^2 + a_{22}(\ln x_2)^2 + a_{33}(\ln x_3)^2 + a_{12}(\ln x_1)(\ln x_2) + a_{13}(\ln x_1)(\ln x_3) + a_{23}(\ln x_2)(\ln x_3)$

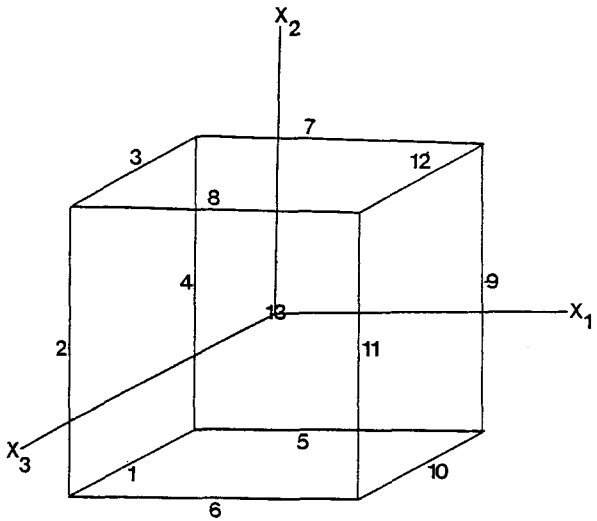

Figure 1. Modified Box and Benhken experimental design.

where y is the response studied and x_1, x_2, x_3 are respectively the mobile phase composition, the flow rate and the column temperature. In our case these variables were coded to have a variation of -1 to +1.

SIMPLEX OPTIMIZATION : To optimize the mathematical model y given by the experimental design, a simplex method was always used. This way, the y value was calculated for m sets of starting condition where m was given by the number of factors to be optimized plus 1. In this case therefore, m was 4. The point corresponding to the lowest value of y was then reflected about the surface defined by the three other points to give a fifth set of starting conditions. Once again, the point with the lowest y was reflected and the process repeated sequentially until an apparent optimum was obtained.

RESULTS AND DISCUSSION

The key chromatographic parameters used as response criteria were the theoretical plate number N, the linear velocity u and the chromatographic resolution function CRF. The data acquired from the design were analysed using software developed in our laboratory.

PLATE NUMBER STUDY : The estimates parameter generated for the regression model are given in table 2. The difference between the predicted and the actual value obtained experimentally was used as a criterion for evaluation of the regression model. This generated model can be assessed statistically (16) using a Fischer Snedecor test (F-test) and the coefficient of multiple determination R^2. The F ratio tests the validity of the model and the value of R^2 is an indicator of the explanatory power model and assumes values from 0, indicating that the variables in the model perfectly explain the variation in the dependent variable N. These criteria were equal to 80, 11 for F and 0.9639 for R^2. This value shows that this model is able to explain a high proportion of the variation present in the theoretical plate number. Calculated values of N are given in Table 3. From the full regression model it was interesting to exclude these variable terms that had no significant effect on the plate number. For this, a student test (T-test) was used to provide the basis for a decision as to whether or not the model coefficients were significant or not. The results of this test are given in Table 4. This reduced model excludes the variable x_2^2 and the interaction $x_1.x_2$. As the matrix of the

Table 2. Regression parameter estimates for the three regression models

Independent variables	Parameter terms	N/L[a]	u[b]	CRF[c]
intercept	a_0	+44.671	+2.747	+36.656
x_1	a_1	-11.641	-0.052	-21.735
x_2	a_2	+5.531	+1.393	+1.021
x_3	a_3	+7.412	+0.006	+1.322
x_1^2	a_{11}	-10.200	-0.021	-1.257
x_2^2	a_{22}	+0.391	+0.285	-1.260
x_3^2	a_{33}	-5.752	+0.023	-0.640
x_1x_2	a_{12}	+0.251	-0.033	+0.536
x_1x_3	a_{13}	-7.052	-0.030	-2.091
x_2x_3	a_{23}	+11.831	-0.011	-1.400

a : inverse of the height to a theoretical plate (mm^{-1})

b : linear mobile phase velocity (mm/s)

c : chromatographic resolution function

Table 3. Response functions calculated for the 13 experiments

Chromatographic conditions							
Experiment N°	Methanol (% v/v)	Flow rate (mL/min)	Temperature (° C)	N/L[a]	u[b]	Fobj[c]	CRF[d]
1	50	0.6	36	41.213	1.636	5.183	55.398
2	50	1.0	50	54.811	2.837	4.940	57.265
3	50	1.6	36	51.780	4.489	5.329	56.352
4	50	1.0	26	25.895	2.765	5.233	55.725
5	63	0.6	26	38.187	1.645	2.349	33.666
6	63	0.6	50	29.358	1.678	2.855	33.822
7	63	1.6	50	64.069	4.443	2.736	33.047
8	63	1.6	26	25.595	4.452	3.091	38.491
9	80	1.0	50	17.437	2.673	0.407	9.610
10	80	0.6	36	17.442	1.599	0.598	10.856
11	80	1.0	26	16.709	2.721	1.456	16.438
12	80	1.6	36	28.991	4.319	1.074	13.953
13	63	1.0	36	44.665	2.747	2.809	36.657

[a] : see Table 2

[b] : see Table 2

[c] : objective function

[d] : see Table 2

Table 4. Student T test used to study the effects of variables of each model

Independent variables	calculated t[a]	
	N/L[b]	u[c]
intercept		
x_1	9.56	4.77
x_2	4.54	127.47
x_3	6.08	0.56
x_1^2	4.48	1.04
x_2^2	0.17	13.92
x_3^2	2.52	1.11
x_1x_2	0.14	2.15
x_1x_3	4.09	1.96
x_2x_3	6.87	0.70

[a]: studentT test

[b] : see Table 2

[c] :see Table 2

experiments is orthogonal the coefficients of the two models are similar thus, it is not necessary to obtain the new coefficients of the reduced model using the two well known "forward" and "backward" methods.

The reduced model excludes the variable x_2^2. This would suggest on the one hand that in the range of variation of the flow rate 0.6-1.6 mL/min, the mobile phase flow rate has no influence on the degree of curvature of the response surface. On the other hand, the non significance of the interaction $x_1.x_2$ shows that it might have been possible to use a univariate approach to optimize these two factors. The retention of a number of second oder terms in the reduced model eg x_1^2, x_3^2, demonstrates that the mobile phase composition and column temperature influence the degree of curvature of the response surface. Thus, for a constant mobile phase composition and flow rate, when the column temperature increases, the solute mass transfer from the mobile phase to the stationary phase increases producing an increase in column efficiency. Over an optimal temperature, the decrease in the capacity factor with the

temperature hides the first phenomena and produces a decrease in the column plate number. By derivation of the model equation, it is shown that the optimum temperature is given by the following equation

$T_{OP} = \exp(-0.85 \ln P + 0.69 \ln D + 7.35)$ [7]

where P (%) and D (mL / min) are the percentage of methanol in the methanol-water mixture and the flow rate.

For p equals 63.24 % and for D equals 0.6 mL/min ; 0.8 mL/min and 1 mL/min, the optimum temperature is respectively equal to 32.22° C; 39.30° C, 45.83° C (Fig.2). According to Horwath and Melander (17) when the percentage of methanol increases the superficial tension between mobile and stationary phases decreases. The consequence is a higher rate of mass transfer of the hydrophobic solute (naphtalene) in the stationary phase and equally in the mobile phase by decreasing the solvent polarity. The factor peak band broadening due to mass transfer decreases. Thus, the plate height decreases. But rapidly, the decrease in the capacity factor with an increase in the percentage of methanol necessitates a higher plate number. In this case beyond an optimal mobile phase composition, this second effect supplants the increase of the column efficiency referred to above and the plate number decreases.

By derivation of the model equation the optimum mobile phase composition is given by the following equation :

$P_{OP} = \exp(0.006 \ln D - 0.246 \ln T + 4.90)$ [8]

For a mobile phase flow rate of 1 mL / min and a column temperature of 26° C, 36° C and 50° C the optimum mobile phase composition was respectively equal to 60.25 %, 55.61 %, 51.30 % methanol in the methanol-water mixture(Fig. 3).

The simplex method was employed to find optimum contitions when the three factors vary simultaneously. The four sets of starting conditions were :

[1] D = 1.00 mL/min T = 30.00° C p = 70.00 %

[2] D = 1.12 mL/min T = 30.47° C p = 71.88 %

[3] D = 1.47 mL/min T = 30.47° C p = 70.47 %

[4] D = 1.12 mL/min T = 31.88° C p = 68.74 %

Twenty five interative processes were performed by computer and the results are given in Table 5. The optimum conditions were a mobile phase flow rate of 1.6 mL/min with a 50.00 %

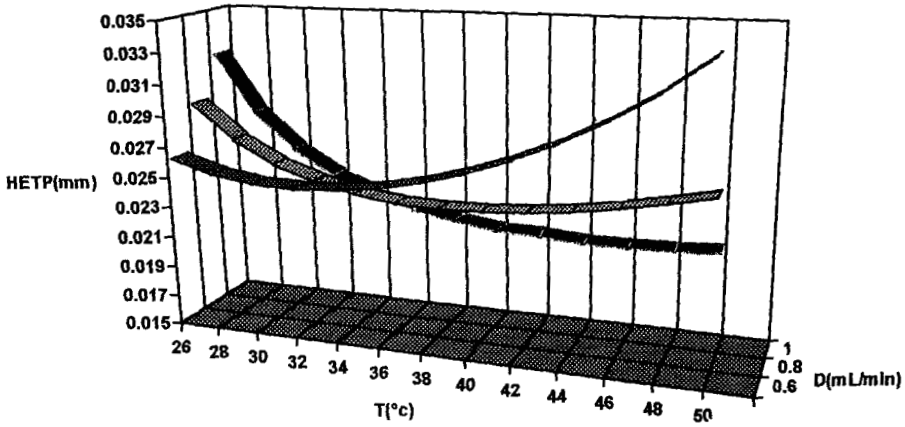

Figure 2. Plots of the height to a theoretical plate vs column temperature (percentage of methanol kept at 63.24 %)

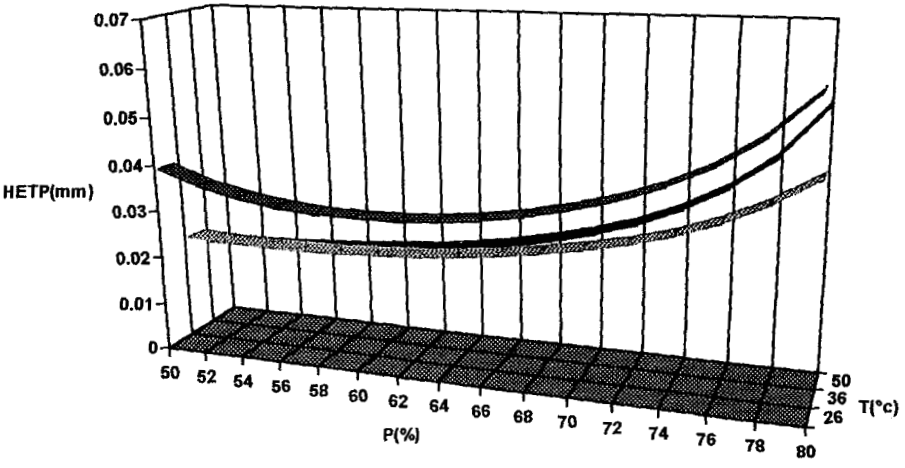

Figure 3. Plots of the height to a theoretical plate vs the percentage of methanol (flow rate kept at 1mL/min)

Table 5. Results of the simplex process for HETP

Experiment N	Percentage of methanol (%)	Flow rate (mL/min)	Temperature (C)	HETP (mm)
1	70.00	1.00	30.00	0.0299
2	71.88	1.12	30.47	0.0314
3	70.47	1.47	30.47	0.0300
4	70.47	1.12	31.88	0.0285
5	68.74	1.27	31.10	0.0277
6	69.00	0.70	31.52	0.0280
7	68.81	1.12	33.02	0.0262
8	67.24	1.00	31.86	0.0263
9	67.52	1.47	32.46	0.0249
10	66.97	1.13	33.78	0.0245
11	68.30	1.48	34.30	0.0236
12	66.39	1.60	34.02	0.0225
13	66.92	1.33	35.61	0.0225
14	65.21	1.23	34.26	0.029
15	65.47	1.31	32.40	0.0243
16	69.83	1.60	33.29	0.2520
17	66.29	1.60	34.83	0.0217
18	61.46	1.60	35.54	0.0196
19	62.76	1.60	40.37	0.0175
20	57.93	1.60	39.07	0.0171
21	59.22	1.60	43.90	0.0157
22	54.42	1.60	42.60	0.0155
23	55.71	1.60	47.44	0.0145
24	50.90	1.60	46.14	0.0146
25	50.01	1.60	49.95	0.0138

percentage of methanol and a column temperature of 50° C. The maximum theoretical plate number was 10846 and the height equivalent to a theoretical plate 0.0138 mm.

LINEAR VELOCITY STUDY : Flow rate varied from 0.6 to 1.6 mL/min. The corresponding average linear velocity u was modeled. The parameter estimates generated for the regression model are given in Table 2. The value of F was 5527 giving an excellent validity for the model. The R^2 value shows that 99.78 % of the linear velocity mean square was explicated. The calculated u values for the 13 experiments are given in Table 3. The results of the student T test are given in Table 4. The mobile phase flow rate had the strongest influence. The retention of the second order term x_2^2 on the reduced model shows that flow rate influenced the degree of curvature of the response surface. For p = 63.24 % and T = 26° C and for a flow rate variation range of 0.6 to 1.6 mL/min the corresponding linear velocity u are plotted on the y axis in Figure 4. The plots show a slight curvature. The reduced model excludes the variable x_1^2 but not x_1 showing that the mobile phase composition did not influence the degree of curvature of the response surface and did not greatly affect the value of u. The terms x_3 and x_3^2 are suppressed from the model. The column temperature does not affect either the curvature of the response surface or the intensity of the value of u. The suppression of the term x_2x_3 from the model would suggest that the interaction is not important. It might have been possible to use a unvariate approach to study these two variables.

CHROMATOGRAPHIC RESOLUTION FUNCTION : As indicated above, the chromatographic resolution function is given by equation [5] where α, β, a are constants which must be determined to have the most efficient CRF to separate the ten benzodiazepines . The constant a is often taken to be equal to 1.4 (10). Calculated values of Fobj (equation [6]) given by the model for the 13 experiments are given in Table 3. The coefficient of multiple determination R^2 corresponding to CRF is given by the well-known equation :

$$R^2 = 1 - \frac{(n-k-1)}{(n-1)} \frac{s_1^2}{s_2^2} \qquad [9]$$

where s_1^2 : residual variance

s_2^2 : Total variance

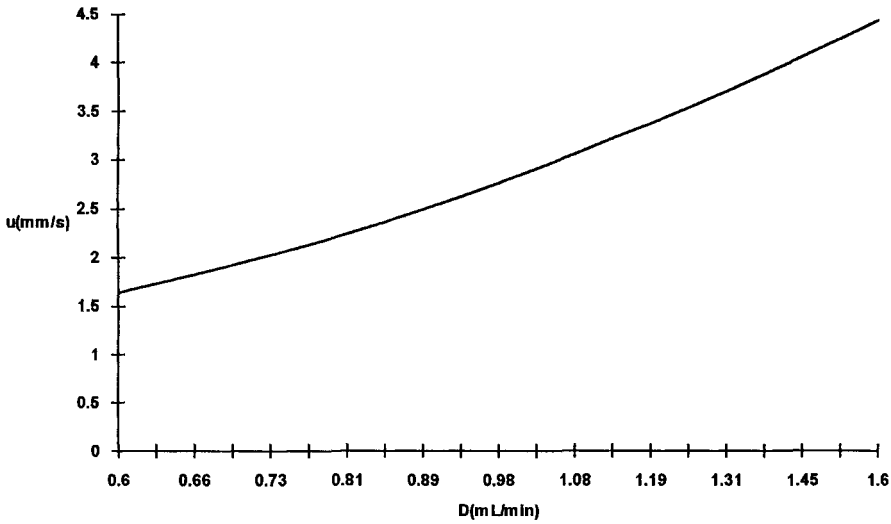

Figure 4. Plots of linear velocity vs the flow rate (percentage of methanol and column temperature kept respectively at 63.24 % and 26 C)

n : N of experiments

k : N of parameters

s_1^2 and s_2^2 are given by the two equations :

$$s_2^2 = 3.1599\,\alpha^2 + 43.5930\,\beta^2 + 21.9389\,\alpha\beta \qquad [10]$$

$$s_1^2 = 0.0285\,\alpha^2 + 1.4795\,\beta^2 + 0.01909\,\alpha\beta \qquad [11]$$

eq [9] is a second order equation in α.

Eq [9] is rewritten as :

$$a\alpha^2 + b\alpha + c = 0 \qquad [12]$$

where

$$a = 37.9188\,R^2 - 37.6624$$

$$b = (263.2668\,R^2 - 263.4386)\,\beta$$

$$c = (523.1160\,R^2 - 509.8005)\,\beta^2$$

Eq [12] has roots if

$$\Delta = b^2 - 4ac$$

Table 6. Results of the simplex process for CRF

Experiment N	Percentage of methanol (%)	Flow rate (mL/min)	Temperature (C)	CRF
1	55.00	0.80	30.00	48.141
2	59.71	0.85	31.18	41.589
3	56.18	0.99	31.18	47.312
4	56.18	0.85	34.62	46.941
5	52.00	0.88	33.71	53.526
6	55.41	1.02	36.82	48.520
7	59.72	1.00	34.95	41.995
8	56.92	0.90	39.01	45.903
9	53.01	0.84	39.03	52.083
10	57.00	0.80	39.20	47.489
11	60.20	0.80	40.00	40.411
12	58.95	0.80	43.02	42.224
13	56.67	0.84	43.22	46.022
14	57.00	0.88	44.23	45.485
15	57.02	0.87	45.81	45.374
16	56.00	0.80	46.00	46.944
17	53.81	0.81	47.80	50.651
18	52.88	0.83	49.10	52.260
19	51.82	0.81	50.00	54.122
20	50.00	0.82	50.00	57.427

is positive. The maximum value to obtain $\Delta > 0$ was $R^2 = 0.9940$

For $R^2 = 0.994$ Roots of Eq [12] are :

$$\frac{\alpha}{\beta} = k$$

where

$k = 6.5071$ or $k = 54.291$

So that the weight of $\sum \ln (1 + dij)$ is not too high in relation to the weight of $N^{1.4}$ $k = 6.5071$

for $\beta = 1$ was used.

$CRF = 6.5071 \sum \ln (1 + dij) + N^{1.4}$

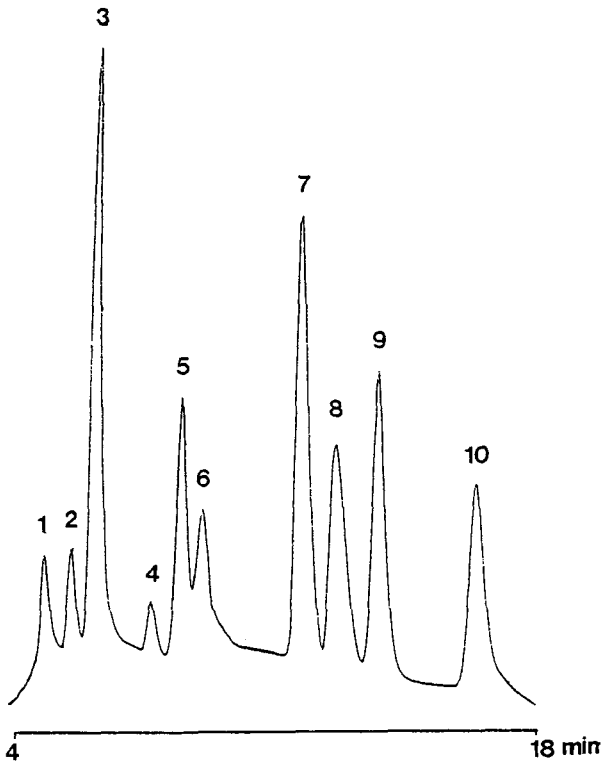

Figure 5. Benzodiazepines chromatogram in the optimum conditions : Methanol = 50 % v/v - Flow rate = 0.82 mL/min - Temperature = 50 C. Number above peaks refers to the benzodiazepines see paragraph reagents and samples.

The parameter estimates generated for the regression model are given in Table 2. CRF calculated values for the 13 experiments are given in Table 3.

In a recent work (9) using another criterion of separation (Ln (1 + Rs) method) it has been demonstrated that the optimum values which gives the most efficient separation conditions are a flow rate of 0.77 mL/min with a percentage of methanol of 49.95% and a column temperature of 51.62° C. In this present work, the highest value of CRF was used as a criterion of separation. Using the sequential simplex method, the four sets of starting conditions were :

[1] D = 0.80 mL/min T = 30.00° C p = 55.00 %

[2] D = 0.85 mL /min T = 31.18° C p = 59.71 %

Table 7. Optimum conditions found with two different methods

Methods	D(mL/min)	T(C)	P(%)
"Ln(1 + Rs)"	0.77	51.62	49.95
"CRF"	0.82	50.00	50.00

[3] D = 0.99 mL/min T = 31.18° C p = 56.18 %

[4] D = 0.85 mL/min T = 34.62° C p = 56.18 %

Twenty iterative processes were performed by computer and the results are given in Table 6. The optimum conditions were a mobile phase flow rate of 0.82 mL/min, a 50.00 % percentage of methanol and a column temperature of 50° C. The maximum value of CRF was 57.427. The chromatogram for these conditions is given in Figure 5. The same optimum values were obtained with these two methods (Table 7) for column temperature and mobile phase composition. The relative deviation for the mobile phase flow rate was 23 %. Optimum column efficiency and optimum separation were only similar in relation to the mobile phase composition and column temperature.

CONCLUSION

Optimizing temperature, flow rate (linear velocity), and mobile phase composition can improve the speed and quality of a separation. The effects of changing column temperature are important as the results of flow rate changes especially if similar compounds are present. It must also be recalled that column efficiency can be greatly improved if column temperature is increased. Agreement between these two separation methods used is good.

Results demonstrate the importance of temperature. Thus, the central design has been shown to be a useful tool for method development when used with a powerful statistical package.

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