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Minimization of baseline disturbances in peptide mapping by means of principal-component multivariate visualization☆

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Abstract

Optimum method parameters for minimizing baseline disturbances in gradient HPLC peptide mapping were identified by the use of principal component mapping. Gradient baseline disturbances were attributed to differences in extinction coefficients between low concentrations of trifluoroacetic acid (0.1%, v/v) in water and in acetonitrile mobile phases. Method parameters that were investigated included (1) column differences, (2) trifluoroacetic acid concentration, (3) mobile phase mixer configuration and (4) detector wavelength. Items (1) and (3) were identified as the most significant method parameters.

1. Introduction

Baseline disturbances in peptide mapping have been attributed to the routine use of 0.1% (v/v) trifluoroacetic acid (TFA) in the gradient water–acetonitrile mobile phase [1]. The difference in extinction coefficient between TFA in water and in acetonitrile produces severe baseline disruptions and loss of sensitivity [2]. In this work, the effect of the following method parameters on baseline disturbances were experimentally investigated: (1) column differences, (2) trifluoroacetic acid concentration, (3) mobile phase mixer design and location on the instrument and (4) detector wavelength. The results of 160 chromatograms representing replicated gradient baselines for 32 experimental conditions were evaluated by the principal-component analysis mapping technique described by Brown and coworkers [3,4].

2. Experimental

The liquid chromatographic systems consisted of a Hewlett-Packard (Valley Forge, PA, USA) Model 1090M instrument equipped with an autosampler and autoinjector, diode-array detector and HP Pascal ChemStation or HP ^{3D}DOS ChemStation. TFA in sealed ampoules (Sigma, St. Louis, MO, USA) was prepared at two concentrations in water (0.1 and 0.05% v/v) and acetonitrile (0.082 and 0.041% v/v). The gradient was run from 0 to 20% acetonitrile in 30 min at 0.3 ml/min and 40°C. The columns

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evaluated were (1) a Separations Group (Hesperia, CA, USA) Vydac C_{18} (250 × 2.1 mm I.D., 5 μm, 300 Å pores) and (2) a Glycotech (Hamden, CT, USA) HY-TACH C_{18} (105 × 4.6 mm I.D., 2 μm, non-porous). Detection wavelengths of 214 and 218-nm with 4-nm bandwidths (214/4 and 218/4 nm notation, respectively) were representative of wavelengths typically used for peptide mapping in the presence of TFA. The reference wavelength was 320/40 nm.

Four types of mixer conditions were evaluated: (1) no mixer (Fig. 1); (2) "double X" mixer (Fig. 2); (3) "ball-bearing" (Fig. 3a and b); and (4) combination of "double X" and "ball-bearing". Baseline chromatograms for a total of 32 experimental conditions were replicated in pentuplicate. Qualitative measurements of baseline height and width disturbances were estimated from visual inspection of replicated chromatograms (Table 1). Multivariate maps of width and height disturbances were prepared using Echo Data (Orem, UT, USA) DataMax software on an 80486DX microcomputer, equipped with Microsoft MS-DOS 6.0, and a Hewlett-Packard LaserJet IIP printer. Using principal-component factor analysis, the DataMax software visually reflects factor relationships by geometrically mapping correlation coefficients.

3. Results

3.1. Baseline chromatogram comparisons

Baseline chromatograms for the two columns, with and without the use of the ball-bearing mixer, are shown in Figs. 4–7. The baseline noise in Fig. 4 for the Vydac C_{18} column without mixer exhibited a periodicity which appeared to correspond closely to the switching of the metering pump valve for the organic phase. Slight periodicity was present in the baseline chromatogram for the HY-TACH C_{18} column (Fig. 6).

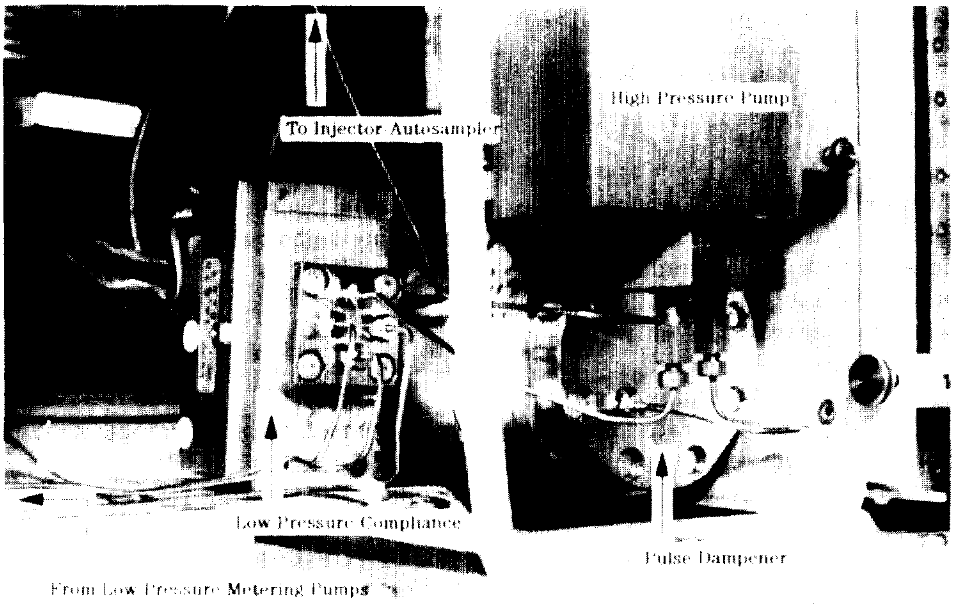

Fig. 1. View of Model 1090 interior with no mixer. The three low-pressure metering pump output lines enter from the lower left and enter the low-pressure compliance. A single output goes from the low-pressure compliance to the inlet side of the high-pressure pump (upper right center). The outlet of the high-pressure pump is plumbed to the pulse damper (lower right center). The outlet of the pulse damper goes to the injector above to the left (not shown).

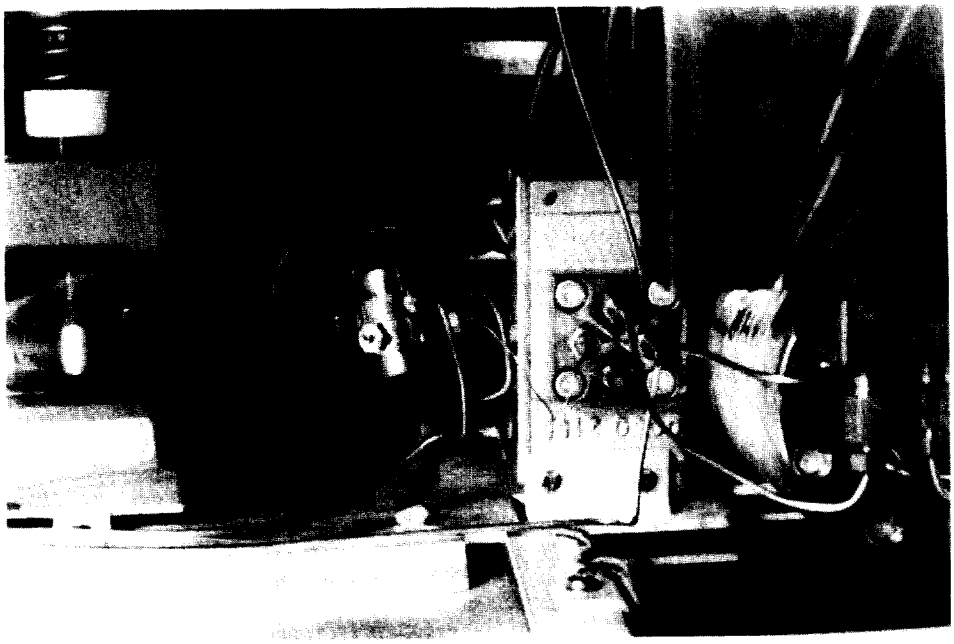

Fig. 2. View of Model 1090 interior with double X mixer. The double X mixer is inserted between the metering pump outputs and the low-pressure compliance. See Fig. 1 for details.

The ball-bearing mixer was found to reduce the periodicity significantly for the Vydac C_{18} column (Fig. 5) and slightly for the HY-TACH C_{18} column (Fig. 7). As the Vydac C_{18} column exhibited the greatest variance, height and width data were analyzed by principal component analysis in order to evaluate the effects, if any, of mixer configuration, TFA concentration and detector wavelength.

3.2. Multivariate mapping

Principal component loadings and scores were mapped for eight Vydac C_{18} experimental conditions (Fig. 8). The resulting centroid map permitted visualization of normalized results for the entire data set. Axes scales were based on normalized differences from the mean with high values corresponding to axes labels. For example, the high, mid-point and low values for the height vector at 2 o'clock were −1, 0 and +1, corresponding to 1.5, 0.6 and 0.31, respectively. The data point with the largest deviation from the mean was plotted at either the high or low ends of the axis. Whereas all four "ball-bearing" experiments clustered at 10 o'clock, corresponding to broad width and small height, the four "no mixer" experiments scattered from 2 to 6 o'clock. It is interesting to note that the lower (0.05%) TFA concentration positioned in the quadrant representing both small height and narrow width whereas a high (0.1%) TFA concentration exhibited a larger noise height.

Principal component loadings and scores for all sixteen Vydac C_{18} experiments are similarly mapped in Fig. 9. Although the "ball-bearing" experiments generally clustered at 10 o'clock, the other experimental results scattered from 2 to 6 o'clock.

4. Discussion

As can be seen from Figs. 4–7, column differences were found to be significant. Both columns were reversed-phase C_{18} and were end-capped.

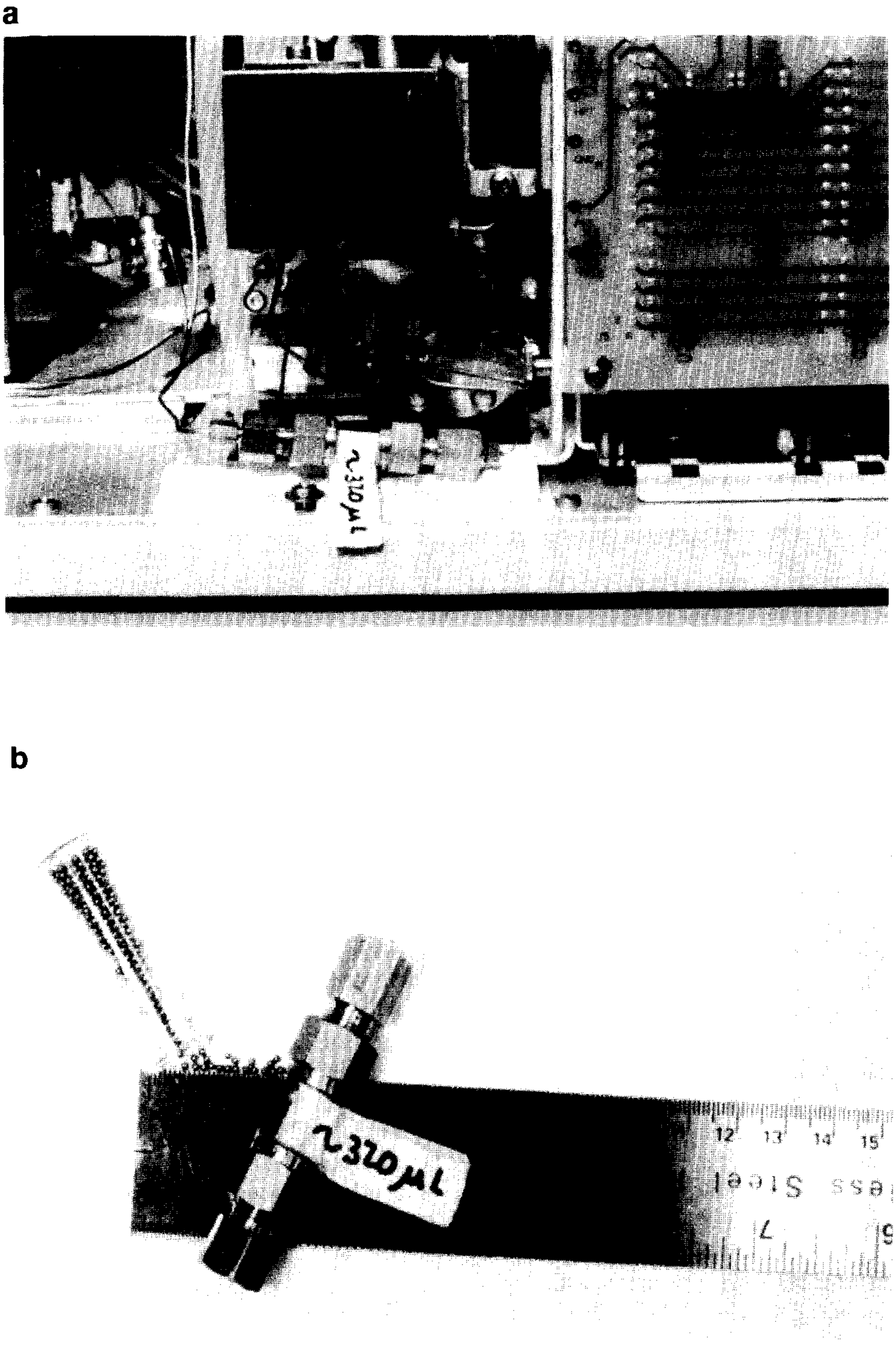

Fig. 3. (a) View of Model 1090 interior with ball-bearing mixer. The ball-bearing mixer is inserted after the pulse damper and before the injector. See Fig. 1 for details. (b) Detail of ball-bearing mixer contents. Ball diameter is 1 mm; mixer dimensions are 4.6 × 40 mm.

Table 1
Vydac C_{18} principal-component results: loading and score results for 8 × 2 matrix comparing the use of no mixer and ball-bearing mixer at wavelengths 214 and 218 nm and at 0.05% and 0.1% TFA with the use of the Vydac C_{18} column (see Fig. 8)

Data point name	Height (mAU)	Width (s)
No mixer 214.1	1.51	35.0
Double X 214.1	0.90	49.5
Ball-bearing 214.1	0.38	73.4
Both 214.1	0.42	72.1
No mixer 218.1	0.86	45.0
Double X 218.1	0.55	47.0
Ball-bearing 218.1	0.31	75.7
Both 218.1	0.29	82.0
No mixer 214.05	0.62	48.0
Double X 214.05	0.65	42.0
Ball-bearing 214.05	0.60	62.0
Both 214.05	0.50	60.0
No mixer 218.05	0.45	42.0
Double X 218.05	0.45	36.0
Ball-bearing 218.05	0.35	72.0
Both 218.05	0.50	60.0

Total variance accounted for:
In one dimension: 12.159%
In two dimensions: 100.000%
In three dimensions: 100.000%

Attribute correlation coefficients:

	Height	Width
Height	1.0000	−0.7568
Width	−0.7568	1.0000

Attribute coordinates:

	X	Y	Z
Height	−0.35	0.94	0.000
Width	−0.35	−0.94	0.000

Communalities:

1D	2D	3D	Sum
0.122	0.878	0.0	1.0
0.122	0.878	0.0	1.0

Object coordinates:

Data point name	X	Y	Z
No mixer 214.1	−0.543	−0.840	0.0
Ball-bearing 214.1	−0.256	0.399	0.0
No mixer 218.1	0.101	−0.305	0.0
Ball-bearing 218.1	−0.235	0.474	0.0
No mixer 214.05	0.366	−0.118	0.0
Ball-bearing 214.05	−0.156	0.100	0.0
No mixer 218.05	0.876	−0.106	0.0
Ball-bearing 218.05	−0.152	0.396	0.0

Only small differences in selectivity and resolution between them have been seen [5]. When a 4.6 mm I.D. Vydac column was used for peptide mixture samples, no differences in selectivity or resolution between it and the 2.1 mm diameter Vydac column were observed; therefore, the column diameter does not appear to be a significant variable in the experiments described here. The baseline deviations present with the HY-TACH column without a mixer were smaller than those encountered with the Vydac column plus both mixers (Figs. 9 and 10 and Tables 2 and 3).

The multivariate maps in Figs. 8 and 9 show that there is a strong clustering of all the results which were obtained with the "ball-bearing" mixer near the broad width and low height portion of the axes (10 o'clock). The "ball-bearing" mixer was very effective in reducing the height and increasing the width of baseline disturbances so that small sample peaks are less likely to be confused with "noise". The baseline "noise" exhibited a periodicity which closely corresponded to the switching of the metering pump valve for the organic phase. The results suggest that an effective mixer must have a volume larger than the stroke volume of the metering pump pistons. In the Model 1090 system, this volume is 100 μl. The "ball-bearing" mixer volume of ca. 320 μl is the lower limit for constructing this shape mixer because of the size of the tube-end fittings.

The observation that lowering the TFA concentration decreases the height but not the width of the disturbances is of definite practical utility as a simple first step that any chromatographer may use. Decreased TFA does not, however, provide the maximum baseline "flatness" exhibited by the use of an appropriate mixer.

The lack of clustering of the variables "double X", 218/4 and 214/4 nm apart from each other and near the broad width and low height portion of the axes is a strong indicator that they are of much less importance in this "noise" reduction experiment.

The fact that the disturbances coincide with the operation of the acetonitrile metering pump switching valve raises the possibility that they are

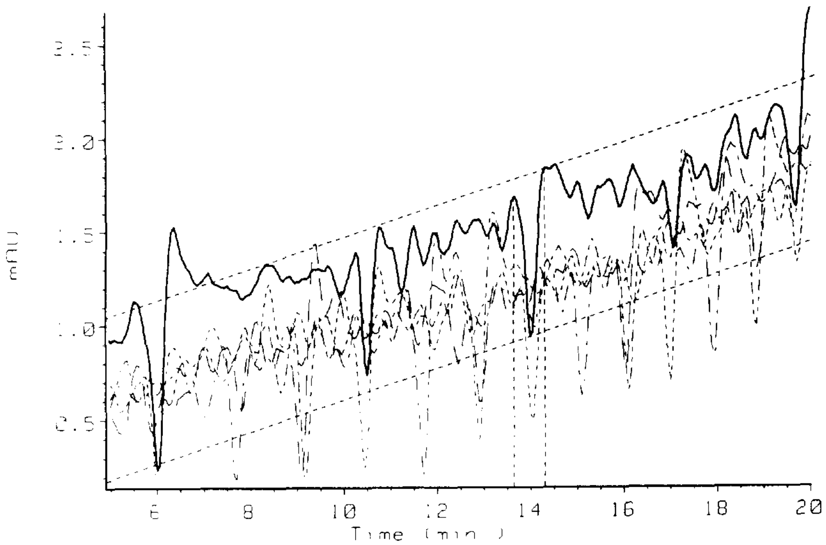

Fig. 4. Baseline disturbances on Vydac column without mixer. Five overlaid traces are shown with one highlighted. The time section of the gradient exhibiting the greatest disturbances is shown. TFA concentration = 0.1% in mobile phase A (water) and 0.081% in mobile phase B (acetonitrile). Gradient conditions as under Experimental. Sample wavelength, 218/4 nm; column, Vydac C^{18}. No mixer was used. Fine dotted lines measure height and width of disturbances.

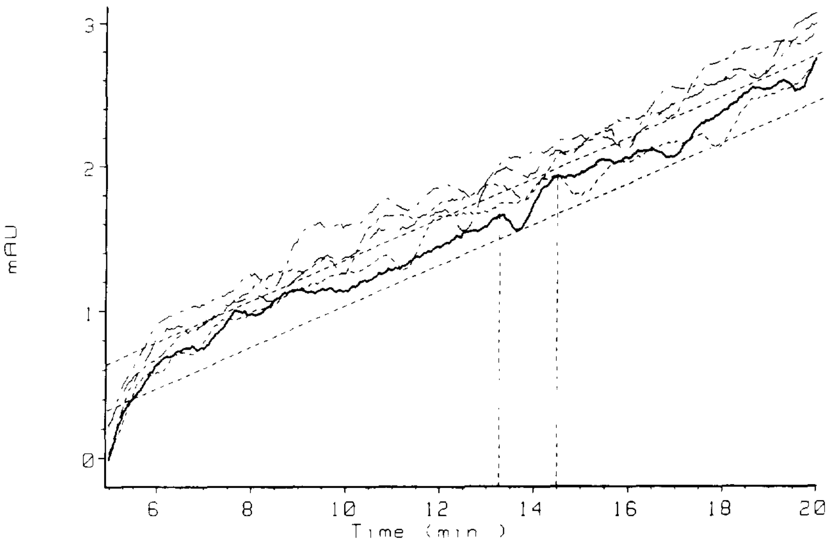

Fig. 5. Baseline disturbances on Vydac column with ball-bearing mixer. Five overlaid traces are shown with one highlighted. The time section of the gradient exhibiting the greatest disturbances is shown. TFA concentration = 0.1% in mobile phase A (water) and 0.081% in mobile phase B (acetonitrile). Gradient conditions as under Experimental. Sample wavelength, 218/4 nm; column, Vydac C_{18}. Fine dotted lines measure height and width of disturbances.

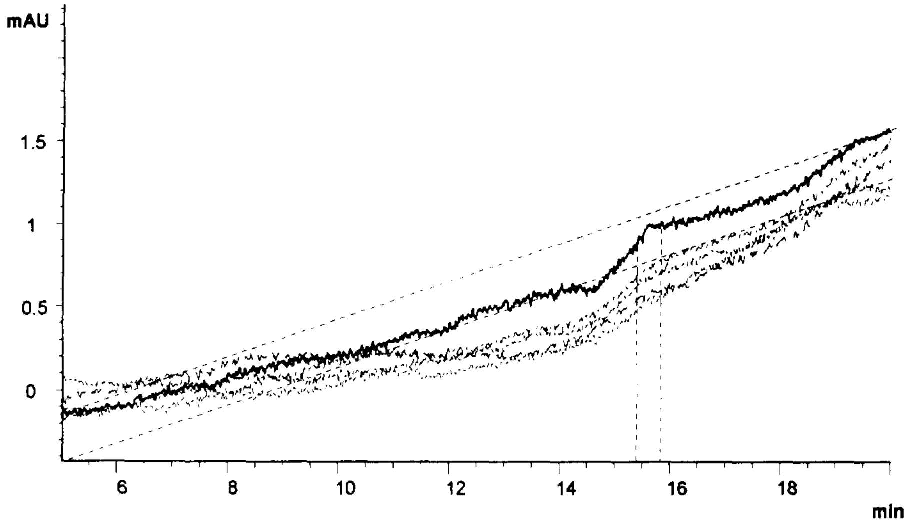

Fig. 6. Baseline disturbances on Hy-Tach column without mixer. Five overlaid traces are shown with one highlighted. The time section of the gradient exhibiting the greatest disturbances is shown. TFA concentration = 0.1% in mobile phase A (water) and 0.081% in mobile phase B (acetonitrile). Gradient conditions as under Experimental. Sample wavelength, 218/4 nm; column, HY-TACH C_{18}. No mixer was used. Fine dotted lines measure height and width of disturbances.

due to the sequestration of TFA in the pores and its release is due to momentary higher water content during the switching cycle. Further study of materials that differ in pore size but not in surface area per gram should help clarify this issue. Study of materials that differ only in the

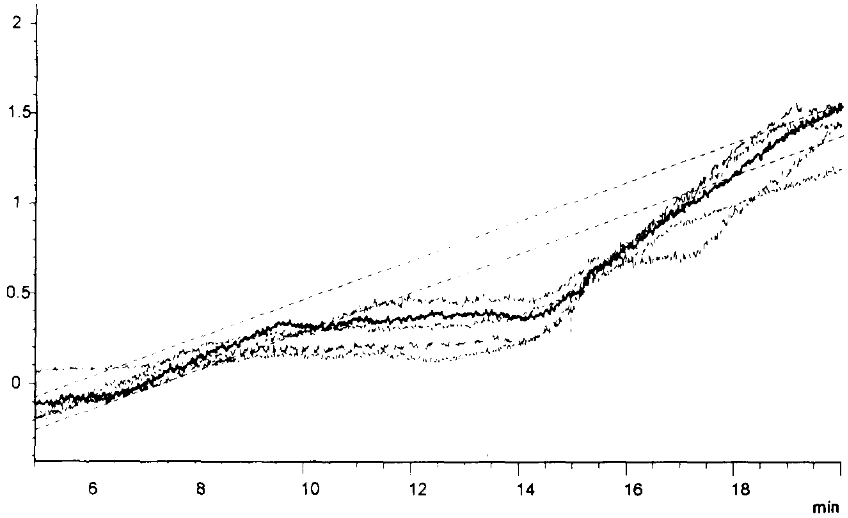

Fig. 7. Baseline disturbances on Hy-Tach column with ball-bearing mixer. Five overlaid traces are shown with one highlighted. The time section of the gradient exhibiting the greatest disturbances is shown. TFA concentration = 0.1% in mobile phase A (water) and 0.081% in mobile phase B (acetonitrile). Gradient conditions as under Experimental. Sample wavelength, 218/4 nm; column, HY-TACH C_{18}. Fine dotted lines measure height and width of disturbances.

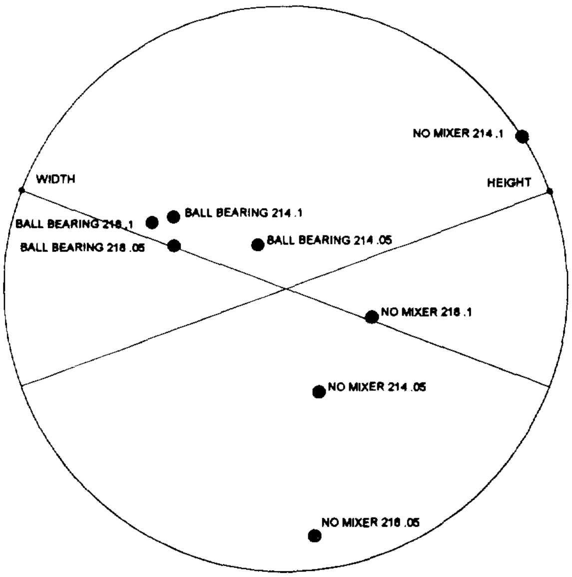

Fig. 8. Vydac C_{18} principal component map (Table 1): comparison of no mixer (upslash) and ball-bearing mixer (black). The four ball-bearing experiments clustered at 10 o'clock (wide width/short height), whereas the four no-mixer experiments scattered from 1 to 6 o'clock. It is interesting that the two no-mixer experiments with 0.05% TFA are positioned in the 6 o'clock quadrant corresponding to narrow width/short height.

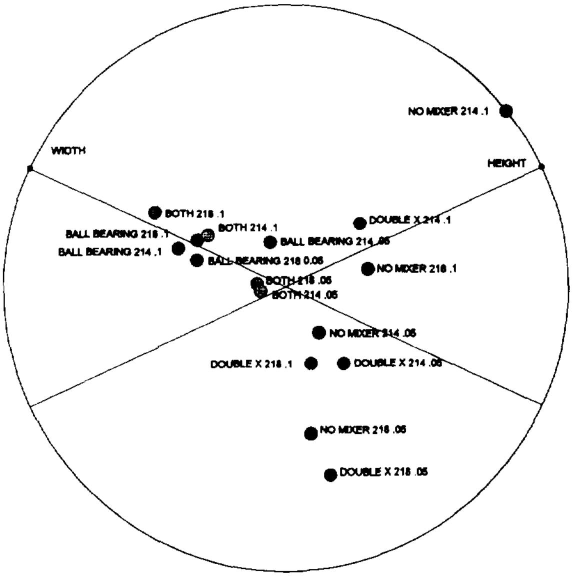

Fig. 9. Vydac C_{18} principal component map (Table 2): comparison of no mixer (downslash), ball-bearing mixer (black), double X mixer (upslash), and both mixers (gray). Results for the double X (upslash) and both (gray) mixer experiments are added to the map shown in Fig. 8. The scattering of the double X mixer from 2 to 6 o'clock indicates no significant effect on minimizing baseline noise. The positioning of both in the same quadrant and ball-bearing is presumably attributable to the use of ball-bearings.

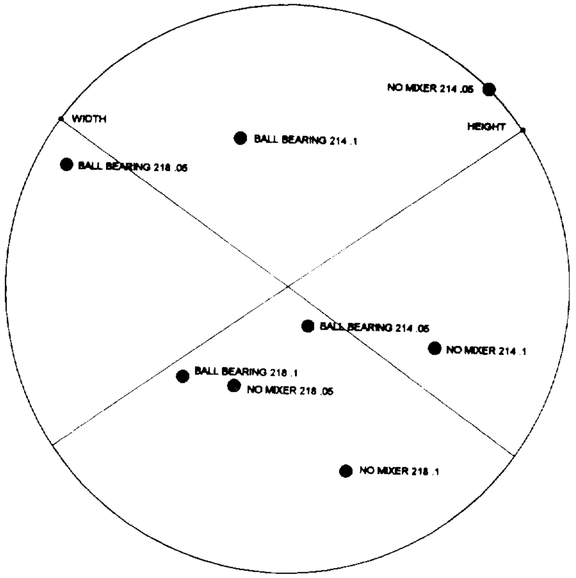

Fig. 10. Hy-Tach C_{18} principal component map (Table 3): comparison of no mixer (upslash) and ball-bearing mixer (black). The scattering of results in this map suggests that the use of the ball-bearing mixer contributed (possibly) to minor improvements in the minimization of baseline noise. This is attributed to the fact that baseline noise was minimal even without the use of a mixer (upslash).

degree of residual surface activity would help in understanding the chemical mechanism of the "TFA effect".

5. Conclusions

The following conclusions can be drawn from the data used in this study: (1) column differences exhibit the strongest effect on the baseline disturbances caused by TFA, but the exact mechanism of how this is achieved is still unclear; (2) a mixer of sufficient volume and efficiency has much more effect than a simple crossover mixer; using a mixer that is larger than the organic phase pump stroke volume is important; (3) lowering the TFA concentration will definitely lower the height of the disturbances; and (4) using a mixer that is significantly smaller than the pump stroke volume has little, if any, effect; and (5) the wavelength of analysis near

Table 2
Vydac C_{18} principal component results: loading and score results for 16×2 matrix comparing the use of no mixer, double X mixer, ball-bearing mixer and both mixers at wavelengths 214 and 218 nm and at 0.05% and 0.1% TFA with the use of the Vydac C_{18} column (see Fig. 9)

Total variance accounted for:
In one dimension: 17.928%
In two dimensions: 100.000%
In three dimensions: 100.000%

Attribute correlation coefficients:

	Height	Width
Height	1.0000	−0.6414
Width	−0.6414	1.0000

Attribute coordinates:

	X	*Y*	*Z*
Height	−0.42	0.91	0.00
Width	−0.42	−0.91	0.00

Communalities:

1D	2D	3D	Sum
0.179	0.821	0.0	1.0
0.179	0.821	0.0	1.0

Object coordinates:

Data point name	*X*	*Y*	*Z*
No mixer 214.1	−0.623	−0.782	0.0
Double X 214.1	−0.223	−0.262	0.0
Ball-bearing 214.1	−0.167	0.314	0.0
Both 214.1	−0.185	0.276	0.0
No mixer 218.1	−0.063	−0.291	0.0
Double X 218.1	0.272	−0.088	0.0
Ball-bearing 218.1	−0.137	0.381	0.0
Both 218.1	−0.267	0.465	0.0
No mixer 214.05	0.161	−0.117	0.0
Double X 214.05	0.271	−0.204	0.0
Ball-bearing 214.05	−0.159	0.056	0.0
Both 214.05	0.014	0.090	0.0
No mixer 218.05	0.519	−0.088	0.0
Double X 218.05	0.667	−0.157	0.0
Ball-bearing 218.05	−0.095	0.315	0.0
Both 218.05	0.014	0.090	0.0

Table 3
Hy-Tach C_{18} principal component results: loading and score results for 8 × 2 matrix comparing the use of no mixer and ball-bearing mixer at wavelengths 214 and 218 nm and at 0.05% and 0.1% TFA with the use of the Hy-Tach C_{18} column (see Fig. 10)

Data point name	Height (mAU)	Width (s)
No mixer 214.1	0.4	30.0
Double X 214.1	0.28	20.0
Ball-bearing 214.1	0.35	120.0
Both 214.1	0.2	45.0
No mixer 218.1	0.25	30.0
Double X 218.1	0.12	60.0
Ball-bearing 218.1	0.16	90.0
Both 218.1	0.1	65.0
No mixer 214.05	0.6	65.0
Double X 214.05	0.4	68.0
Ball-bearing 214.05	0.3	67.0
Both 214.05	0.30	120.0
No mixer 218.05	0.20	75.0
Double X 218.05	0.20	67.0
Ball-bearing 218.05	0.18	160.0
Both 218.05	0.16	160.0

Total variance accounted for:
In one dimension: 32.737%
In two dimensions: 100.000%
In three dimensions: 100.000%

Attribute correlation coefficients:

	Height	Width
Height	1.0000	−0.3453
Width	−0.3453	1.0000

Attribute coordinates:

	X	Y	Z
Height	−0.57	0.82	0.000
Width	−0.57	−0.82	0.000

Communalities:

1D	2D	3D	Sum
0.327	0.673	0.0	1.0
0.327	0.673	0.0	1.0

Object coordinates:

Data point name	X	Y	Z
No mixer 214.1	0.203	−0.527	0.0
Ball-bearing 214.1	−0.521	0.181	0.0
No mixer 218.1	0.639	−0.223	0.0
Ball-bearing 218.1	0.322	0.364	0.0
No mixer 214.05	−0.717	−0.697	0.0
Ball-bearing 214.05	0.136	−0.075	0.0
No mixer 218.05	0.350	0.182	0.0
Ball-bearing 218.05	−0.412	0.795	0.0

the "pseudo-isosbestic point" of 214–218 nm does not matter for "noise" reduction. The only influence that is easily noticeable is a reduction of baseline rise with increasing acetonitrile content at some wavelengths.

References

[1] C. Woodward, poster presentation at the 13th American Peptide Symposium, Edmonton, Canada, 1993.
[2] G. Winkler, P. Wolschann, P. Briza, F.X. Heinz and C. Kunz, *J. Chromatogr.*, 347 (1985) 83–88.
[3] F.O. Geiser, C. Golt, L. Kung, Jr., J.D. Justice and B.L. Brown, *J. Chromatogr.*, 631 (1993) 1–13.
[4] B.L. Brown, R.N. Williams and C.D. Barlow, *J. Pascal, Ada, & Modula-2*, March/April (1984) 19–24.
[5] C. Woodward, unpublished observations.