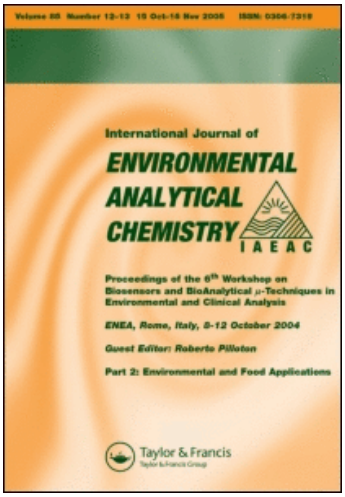

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Increased Flexibility in Multicolumn Chromatography (MC–HPLC) via On-line Effluent Mixing Technique*

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Dedicated to Professor W. Haerdi on the occasion of his 60th birthday

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In continuation of our work dealing with multicolumn HPLC (MC–HPLC) we describe in this paper an on-line on-column fraction trapping technique based on effluent mixing.

To a normal two-column switching set-up (in this case with two RP columns) an additional high-pressure pump gets inserted into the connection line between column A and column B via a low dead volume mixing tee. The in-line respectively off-line switching of pump B and the mobile phase B is time controlled by using a high pressure switching valve. With this set-up it is possible to mix on-line an effluent fraction from column A and transferred onto column B with a highly polar and pH-controlled (e.g. aqueous buffer) new effluent, to reduce or adjust significantly the overall elution strength of this mixed transferred solvent. Thus, several chromatographically effective possibilities can be created in a simple manner, which are for example: (a) pronounced peak compression respectively on-column concentration on column B; (b) due to low elution strength and/or pH adjustment during the trapping period on column B, increments to the overall selectivity of the column switching set-up can be

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added creating multidimensionality via mobile phase switching; (c) combining the heart cut with the effluent mixing technique enables analysis of trace peaks eluted on the back flank of an overloaded main peak.

KEY WORDS: Multicolumn HPLC, multidimensional HPLC, fraction trapping, effluent mixing.

INTRODUCTION

Since the late sixties, when D. Deans[1] has proposed column switching as a valuable tool for trace analysis in GC, and later J. F. K. Huber and co-workers[2] could show the great potential of multidimensional HPLC, the latter technique became quite popular. Not all work that is published on this subject is truly "multidimensional", however, the various applications of column switching or multicolumn chromatography (MC–HPLC) have proven its usefulness. More than a hundred relevant papers on MC–HPLC have been published yet, and the applications of environmental trace analysis and in particular in the biopharmaceutical field including on-line pre-column sample pretreatment are numerous. Review articles on MC–HPLC are rare, however contributions should be mentioned in this context.[3-7]

D. Freeman[8] expressed mathematically the enormous enhancement of selectivity to be gained with column switching and mode sequencing and Guiochon *et al.*[9] contributed on the selection of optimal combination of systems for two-dimensional chromatography, but not much attention to the fact of possible incompatibilities of coupled systems has been paid. Also and this had been mentioned by several others,[10-20] it is important to perform so-called on-column concentration effects in column switching set-ups. This means, that in an on-line coupled two-column or two-systems set-up (A and B) the chromatographic conditions have to be selected in a way to allow the injection or the transfer of a rather large solvent volume onto column B corresponding to a rather large transfer volume (e.g. zone or heart cut) eluting from system A and to be loaded onto column B. Without the performance of on-column concentration effects on column B large injection volumes (e.g. $>200\,\mu l$ on conventional >4 mm i.d. analytical columns) would lead to intolerable bandbroadening, subsequently reducing the high

efficiency of system B and thus also of the total MC–HPLC system. Consequently, the sequence as well as the type of columns or systems to be coupled on-line becomes important.

Also the solvent (mobile phase) fractions to be transferred on-line from system A to system B have to be compatible. Only systems running in aqueous or non-aqueous modes may be coupled directly, otherwise an interface has to be incorporated between the incompatible systems. This possibility was shown by Sonnefeld *et al.*[21] who trapped an organic fraction onto a concentrator column and blew out the solvent with nitrogen gas; afterwards it was ready for reversed phase elution. However, such MC–HPLC set-ups need much hardware and are not always easy to handle.

In some cases it might also be possible to switch, via an intermediate column, between two immiscible mobile phases by stepwise running intermediate solvent mixtures which are compatible and assuming that the compounds of interest get trapped on the intermediate column and stay strongly retained during the stepwise change of mobile phases.

Generally, column coupling in reversed phase mode is dominant, whereby the sequence of the coupled columns or systems should be the following: aqueous size exclusion and/or ion exchangers—moderately polar or lipophilic phases and/or ion exchanger—non-polar, highly lipophilic phases.

With such arrangements and at a given elution strength of a mobile phase the retention of an ionizable or medium polar analyte will increase. Subsequently, the eluting effluent from column A carrying the compound(s) to be transferred to column B will have a weak elution strength on the following system. Peak compression or on-column concentration effects on the top of this column (B) occurs. Its magnitudes, especially when running pure aqueous mobile phases, and in dependence of the type of solutes and stationary phases, have been estimated and measured by several authors.[22–25]

In this paper we discuss techniques of coupling columns which are relatively similar in terms of their lipophilic characteristics, e.g. two reversed phase RP18 columns. Coupling them in the conventional way (in series), the only effect would be the enhancement of the total plate number N due to the increased column length, but very little increment on chromatographic selectivity will be observed. Also no on-column concentration effects occur on the top of column B by

transferring an effluent fraction from system A onto B, since both columns need mobile phases with equal elution strength. However, by weakening the elution strength of the effluent fraction to be transferred from column A to column B by on-line mixing the fraction volume with a very aqueous solvent prior to its loading onto system B via an external pump, significant on-column focusing effects and changes in retention characteristics, respectively, of the transferred compounds can be obtained. Following the trapping on column B by a simple (step) gradient elution of column B without changing the selectivity of the mobile phase, corresponding to a (step) gradient elution profile run on the in-series coupled columns A and B, would lead to relatively similar chromatograms in terms of resolution. However, by this on-line effluent mixing the selectivity of the mobile phase of the transferred effluent fraction can be changed (e.g. pH change) in a very simple manner with the result, that the overall selectivity of system B is different from system A even when the columns A and B are similar. Thus, only via an on-line mobile phase variation by effluent mixing during the period of the fraction transfer (heart cut) from column A onto column B an increment to the overall selectivity of the two column systems can be created. This can truly be different to a normal (step) gradient elution profile of serially coupled columns A and B. Potential applications of this effluent mixing technique used in on-line multicolumn chromatography are discussed.

EXPERIMENTAL

Column switching apparatus

The set-up was a modular system consisting of two pumps Model 410, column and solvent switching unit Model Tracer MCS 670, programer Model 200, variable wavelength detector Uvicon 720 LC (all models Kontron, Switzerland). The two similar columns were packed with Spherisorb ODS, 5 μm, 100×4.6 mm i.d. (Brownlee, USA). The solvents used for chromatography were p.a. grade from Merck (West Germany), the water was bidistilled in a quartz apparatus. The test compounds, ioxynil and bromoxynil, were donated by Chemie Linz AG (Austria).

RESULTS AND DISCUSSION

The basic column switching set-up is depicted in Figure 1. It is a normal configuration except of the installation of a low dead volume mixing tee, by which additional solvents may be pumped to the effluent stream coming from column A and directed to column B or via a bypass (valve V3) directly to the detector. The time the solvent mixing step is activated is only controlled by switching valve V2 back and forth and presetting the appropriate flowrate of pump B. For better controlling respectively adjusting, the absolute flow of pump B, it is advised to run pump B always against a pressure restrictor or dummy column during the non-activated mixing period, otherwise the adaptation to the actual stipulated flow of column B could be relatively slow when activating the mixing step, depending on the type of pumps (A and B) and the devices of controlling and adjusting the actual flow in connection with back pressure changes.

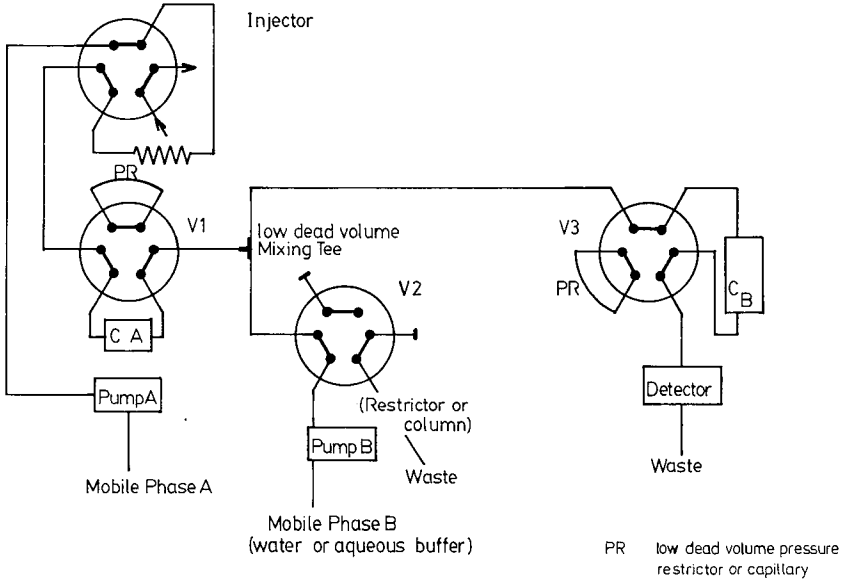


Figure 1 Basic valve and column switching configuration involving the effluent mixing technique used for trace analysis of ioxynil besides large excess of bromoxynil (see Figure 7). Columns A and B: 100 × 4.6 mm i.d., packed with Spherisorb ODS, 5 μm.

However, this is not critical as long as it is taken in account according to the setting of a time window of the zone cut time, which is probably prolonged due to an initially decreased flow of mobile phase A (caused by the increased column length which is now A plus B) on column A.

To evaluate the system parameters for the effluent mixing technique we used the herbicides ioxynil and bromoxynil, as moderately polar test solutes (structure, see Figure 2). Chromatograms of these compounds run under isocratic conditions on column A and column A + B, connected in series, are shown in Figures 3a and 3b; doubling the column length gives the expected results in terms of plate number and resolution in consideration of a significant contribution of external band broadening, due to the rather large dead volumes of capillary connections and valve units. The injection volume onto column A was 20 μl for this experiment. The resolution R of the main peaks increased from 1.1 to 1.9.

In the following experiment, we activated the aqueous effluent mixing solvent stream (pump B) via valve switching of V2 during the indicated zone (see Figure 3a), whereby the flow rate of pump B was pre-set to 2 ml, which gives a total flow (pump A plus pump B) of approximately 3 ml during the trapping period. Partial peak compression of ioxynil and bromoxynil on column B occurs. Switching back valve V2, the initial mobile phase A enters the column B. A steep step gradient is now performed in column B, since the column B was, for a defined period of time respectively total effluent volume, as a consequence of the effluent mixing step, treated with a highly polar mobile phase. As a result, see Figure 3c, one gets baseline separation of the initially unresolved impurity peaks (see Figure 3b)

Figure 2 Formula scheme of the test compounds.

and an increase of the resolution between bromoxynil and ioxynil from 1.9 to 2.5.

In principle, this result is not unexpected and one observes relatively similar chromatograms, if column A or the coupled columns A + B were run in a normal (step) gradient mode (chromatograms not shown). The increase of resolution R by (step) gradient elution is mainly attributed to the fact, that the selectivity changes slightly between ioxynil and bromoxynil with the mobile phase composition or the percentage of the organic modifier in the aqueous acidic mobile phases. Indeed, this behavior was found to be realistic, as may be seen in Figure 4; the k' characteristics of the two test compounds as a function of the methanol content of the mobile phase behave non-parallel to each other, and are also non-linear at low percentage of the organic modifier. Consequently, the resolution R changes with the methanol content, as a trend R increases with the decrease of methanol percentage, which is not exceptional in RP–HPLC, it is rather the rule.

However, a normal (step) gradient elution pattern is not the point of this particular column switching set-up with an effluent mixing device. It is rather the fact using coupled column chromatography together with a heart cut or zone cut transfer from column A to column B, involving the trapping of the zone onto column B, in order to perform a particular gradient elution analysis of only a relatively small part of the total chromatogram. With the on-line dilution of the mobile phase the transferred zone from column A is trapped, respectively eluted with a steep "negative" gradient on column B, which results in a significant slowing down of the migration of the peaks (zones) and which is not necessarily equal for all compounds (see k' and R as a function of the elution strength of the mobile phase).

Depending on the mixing rate of mobile phase A to the aqueous diluter (mobile phase B) the elution strength of the mixed mobile phase is adjusted during the transfer time and with it the degree of retention (compression) of the compounds on column B. This is demonstrated by Figure 5 which also shows that with the increase of the trapping time the finally observable resolution R of the pair of peaks can be influenced to some extent. The longer the weak (slow) eluting mobile phase is activated, the better the resolution becomes. The slowed down migration via effluent mixing is followed by a

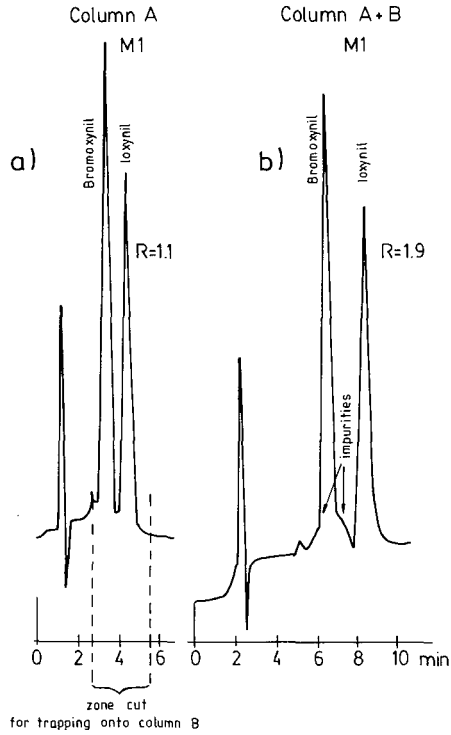


Figure 3 Separation of ioxynil and bromoxynil: (a) on column A; (b) on column A + B, connected in series and run isocratically; (c) on column A and B, but with fraction trapping of the zone cut from column A onto column B.

MC–HPLC set-up (see Figure 1); mobile phases (M):

MA) H_2O-acetic acid–methanol/45-1-55;
MB) H_2O-acetic acid/100-1;

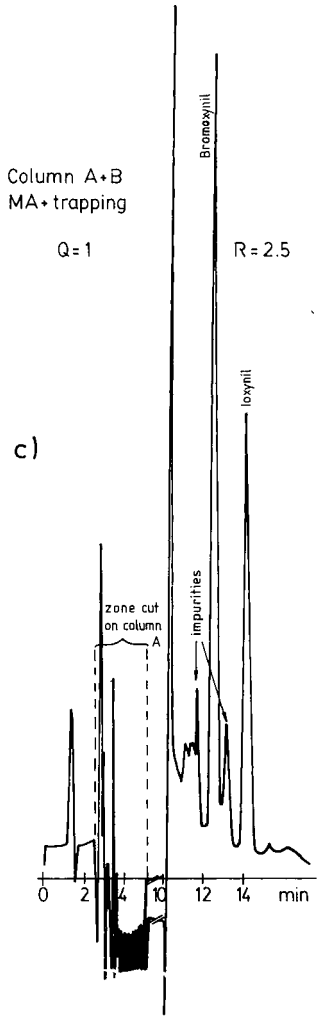
flowrate of pump A (PA) and pump B (PB) 1 ml/min each.

Time diagram of function activated:

zone cut of column A trapped onto column B

	0 (min)	4	8	12
Column A	PA–MA		off-line	
Column B	off-line	PA–MA + PB–MB	PA–MA	

Trapping quotient $Q = \frac{\text{ml flow PB–MB}}{\text{ml flow PA–MA}}$.



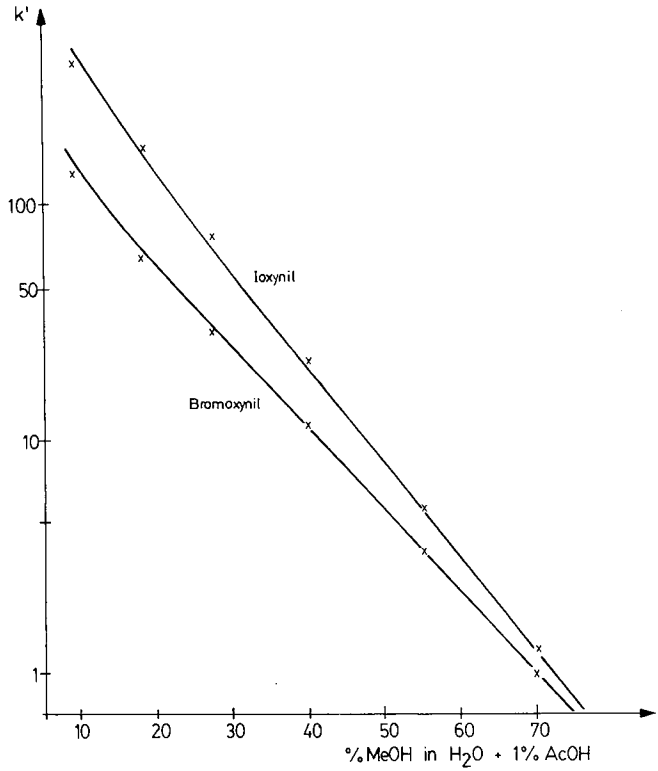


Figure 4 Log k' values of ioxynil and bromoxynil as a function of the methanol content of the aqueous mobile phase measured on a RP18 guard column 30 × 4.6 mm i.d., packed with Spherisorb ODS, 5 μm.

steep "positive" gradient elution of the trapped, respectively slowed down, zones in form of sharp and resolved peaks. The increase of resolution by trapping (see Figure 4 and above) is obviously not completely eliminated by the gradient elution, which leads to an overall increased selectivity paired with the advantage of a normal (step) gradient elution chromatography. Somewhat similar effects were observed by Berry[26] using a "pulsing" technique with weak eluent plugs to increase the resolution of badly resolved peaks.

But besides the effect of increasing the selectivity and thus also the resolution of given pairs of peaks, the zone cut and transfer

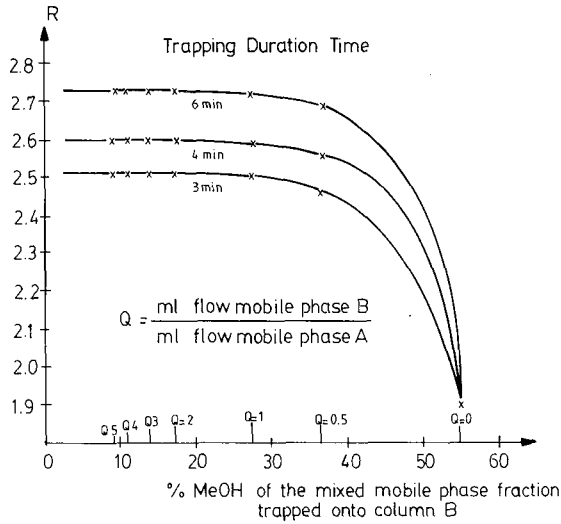


Figure 5 Resolution (R) of ioxynil and bromoxynil as a function of trapping Q and of the total trapping duration time. Columns, mobile phases and MC–HPLC set-up (see Figures 1 and 3).

combined with the effluent mixing technique offers another and most significant advantage, which is demonstrated by the following example, the determination of 0.3% ioxynil besides 100% bromoxynil, whereby the trace compound ioxynil is eluted after the main compound, which is usually very disadvantageous. As expected and shown in Figure 6a, to determine a small peak on the back flank of a heavily overloaded peak (peak size ratio 1000:3) the resolution factor R of this pair of peaks has to be increased substantially (compared to $R=1$ and a peak size ratio of 1:1 or 10:1) in order to ensure baseline separation and subsequently sufficient quantification. To achieve such high selectivity might be quite cumbersome and could be problematic in terms of sensitivity of the trace peak due to prolonged retention time. The usual way to get around this problem would be to find a chromatographic system with a reversed elution order (the trace component elutes in front of the main peak), but this is often not so simple.

However, if this approach is not successful one could try to get a more favorable peak size ratio (e.g. 10:1 or better) via a zone cutting

procedure with multicolumn chromatography. Hence, the above-mentioned problems could be overcome to some extent.

In order to make this possible, only a small portion of the first eluting main peak (back flank) and the trace peak should be transferred from column A to B and trapped on it. In reality several hundred μl up to ml fraction volume are transferred respectively injected onto column B which is chromatographically only acceptable if a pronounced on-column concentration effect of the compounds transferred is performed.

Through the effluent mixing technique described in this paper this goal can be achieved to a great extent. With the example shown in Figure 6b it becomes obvious, that this selective trapping technique enables a trace analysis of an impurity (0.3% ioxynil) eluted after the main peak, which is commonly only realistic, as mentioned before, when the trace peak is eluted in front of the large peak.

As can also be seen from Figure 6b, by this column switching and fraction trapping method, some impurity peaks from the bromoxynil standard could be traced and perfectly separated (compare also Figures 6a with 3b and 3c) due to the increased overall selectivity of the HPLC system.

That the peaks are real substance peaks and not artificial due to some unwanted phenomena (peak splitting, etc.), we characterized the peaks by on-line diode array UV spectra and found all peaks to be chemically and structurally different (data not shown).

CONCLUSION

The described effluent mixing technique in multicolumn (column switching) chromatography (MC–HPLC), especially in the reversed phase mode, offers several features:

a) MC–HPLC becomes more flexible in respect to column sequencing, since fraction compression steps are relatively simple to perform on the following system.

b) The selectivity, and subsequently the resolution of a given pair of peaks can be increased due to the sequence of steps of "negative" and "positive" (step) gradient elution. This gain on on-line adjustable selectivity has to be paid by an increase of the overall analysis time. It is prolonged for the factor of trapping time.

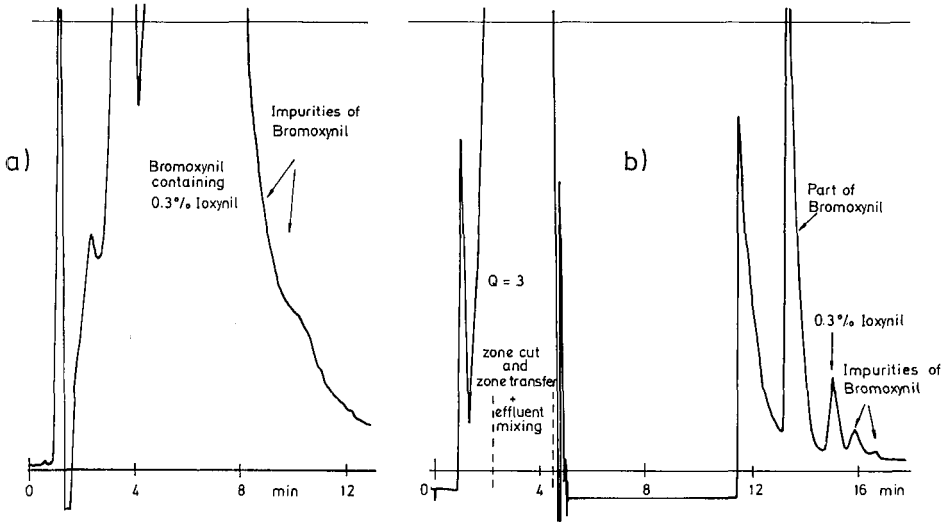


Figure 6 Separation of traces of ioxynil besides a large excess of bromoxynil: (a) on column A and B connected in series and run isocratically with mobile phase A; (b) after zone cutting on column A and fraction trapping via effluent mixing on column B. Columns and mobile phases (see Figure 3); MC–HPLC set-up (see Figure 1).

Time diagram of function activated:

zone cut of column A trapped onto column B

	0		4		8	12
	(min)					
Column A	PA–MA				off-line	
Column B	off-line	PA–MA + PB–MB			PA–MA	

Note: The time window of the zone cut has to be activated earlier than in Figure 3 indicated. This comes due to sample overloading effects on column A subsequently changing the retention characteristics.

c) Through the effluent mixing technique one creates peak (fraction) compression, also the speed of the peak migration through the column slows down drastically, but which is often not zero (see b). If it is close to zero, one gets a real on-column concentration effect which could be used for multiple effluent trapping; a valuable tool in trace analysis.

d) The trace detection on tailed back flanks of overloaded peaks becomes practicable via the combination of zone cutting with the effluent mixing technique.

e) Using aqueous buffers as effluent mixing solvents, it is feasible to change the pH values of the fraction to be transferred and trapped. Thus, it is possible to split on-line a sample matrix into, e.g. ionizable basic and acidic compounds by retaining both groups separately on different pH adjusted RP systems.

f) The reproducibility of the system in terms of retention time and peak size is comparable to a normal (step) gradient elution method, but relays also on the technical reliability of the pumps and switching units (e.g. flow control, time controlled valve switching).

g) In principle, this technique is not restricted to reversed phase systems, it should also work in non-aqueous systems, although we have not tested it yet.

All the practical experience we have gained so far with effluent mixing in MC–HPLC[17,18] convinced us, that it is a valuable technique to use in routine work without any problems, as, e.g. clogging of column frits or significantly reduced life-time of the columns. This might often be attributed to poor column packing rather than multiple gradient usage.

Finally, it should also be noted, that this MC–HPLC technique needs more hardware than normal HPLC systems; however, the advantage in terms of flexibility to set-up trace analysis MC–HPLC systems which have been shown to work on a routine basis, justifies the additional equipment.

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