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SEQUENTIAL DIFFERENTIAL DETECTION IN LIQUID CHROMATOGRAPHY

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SUMMARY

The use of differential detectors in chromatography requires that the sample chamber and the reference chamber are properly balanced in the absence of the analyte. We show that this condition is most readily satisfied if the two halves of the differential detector are used in series in the effluent stream, separated by a fixed delay loop. The conversion of the measured differential response to a normal chromatogram is trivial. Several examples of this scheme applied to refractive index and to absorption measurements in liquid chromatography are reported here.

INTRODUCTION

The precision and the sensitivity of analytical measurements are generally enhanced when a differential response is obtained between a sample chamber and a reference chamber. The effects of the "matrix" can then be cancelled out so that the response of the analyte can be magnified. This is particularly true in liquid chromatography (LC), where the eluent contributes substantially to the background level of the detector in many cases. It is therefore not surprising that most of the commonly available commercial detectors for LC are based on differential schemes.

In practice, however, it is very difficult to achieve a true cancellation of the contributions of the LC eluent even using a sample and a reference chamber. The reason is that the two chambers are effectively isolated from each other so that the environment in one chamber is always slightly different from the other. The inability to reproduce the conditions in the two chambers is then the primary limitation in differential detectors. The most obvious example is the refractive index (RI) detector in LC. It is known that detectability is eventually limited by temperature and pressure fluctuations in the LC effluent[1]. Since the reference cell in the RI detector is typically used in the static mode, complete equilibration of temperature and pressure relative to the sample flow cell is unlikely. It is in principle possible to split the eluent before the point of injection to be used in the reference cell, with or without an intervening matching column. Even so, the elution process in the analytical column can itself lead to differences in temperature and pressure in the sample flow cell. The extreme

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case is that of gradient elution, when one must balance the sample and the reference cells to the order of the RI change of the analyte. It is apparent that the same limits exist in the absorption detector in LC. There, fluctuations in the RI can affect the amount of light reaching the photoelectric transducer, resulting in false absorption signals. Also, if the eluent absorbs light, due to a natural absorption band or due to bleeding of the column, pressure fluctuations cause fluctuations in the number of molecules in the optical path, and thus the background is affected.

One solution to this problem is to use differential detectors in series along the chromatographic effluent stream. It is clear that since the two chambers are connected, pressure and temperature differences can be minimized. A series arrangement for differential detectors in LC has been reported earlier[2], but, as we shall see in what follows, there are substantial differences in our findings *versus* theirs. Two possible arrangements are shown in Fig. 1. The sample (S) and the reference (R) flow cells are separated by a delay loop (D) of known volume, V (ml). In (a) the detection chambers are truly in series while in (b) they are effectively in series. One can see that in either arrangement, a response in the sample cell at any given elution volume, V', during the separation will give an equal but opposite response in the reference cell (opposite because of the differential nature of the detector output) at an elution volume of $V' + V$. If the eluent flow-rate is F (ml/min), this corresponds to a time delay of V/F (min) between the equal but opposite signals of the same event to register at the detector. So, to produce chromatograms that resemble conventional detection methods, one can simply add to the detector signal at any given instant the particular detector signal recorded exactly V/F min earlier in the separation. The conversion is simple enough to be handled by a microprocessor in real time. Further, unlike ref. 2, this conversion procedure does not require the assumption of any particular band shape in the chromatograms. The concept of sequential differential detection thus allows the proper equilibration between the sample and the reference cells of a differential detector, while retaining all of the chromatographic information.

A closer examination shows that there are in fact some guidelines towards the

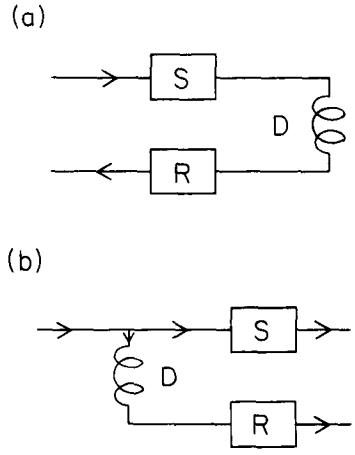


Fig. 1. Two arrangements for differential detectors: truly in series (a); effectively in series (b). S = Sample cell; R = reference cell; D = delay loop.

choice of the delay volume, V. If V is too large, band broadening within the delay loop destroys the simple correlation between the responses in the sample cell and the reference cell. If V is too small, the net signal obtained for any chromatographic event will be reduced, since the response then becomes a true derivative of the normal chromatographic signal. Sensitivity in the measurement is then sacrificed. A reasonable choice is then a volume in the order of half the elution volume of a typical chromatographic peak under the particular conditions for separation. In fact, since the differential detector is now properly balanced, detectability may even be improved.

It is important to note that this scheme is advantageous only if the detector is truly differential in nature, *e.g.*, the RI detector and the absorption detector in LC. Otherwise, one will simply be correcting a large signal with another large signal, which is neither practical nor justifiable.

EXPERIMENTAL

Chromatography

All reagents and eluents used were reagent grade material without further purification. Separation was performed on a 25 cm × 4.6 mm I.D. 10-μm Alltech C_{18} column (600 RP, Alltech, Deerfield, IL, U.S.A.). Samples were eluted with pure acetonitrile as the mobile phase. All injections were made through a 5-μl sample loop at a conventional injection valve (Model 7010; Rheodyne, Berkeley, CA, U.S.A.). Solvent delivery is from a metering pump (Model 750; Micrometrics, Norcross, GA, U.S.A.) operated in the constant volume mode. Studies of gradient elution were performed using either a commercial gradient programmer (Model 980A; Tracor, Austin, TX, U.S.A.) ahead of a reciprocating pump (Model 196-0066; Milton Roy, Riviera Beach, FL, U.S.A.), or a high-pressure mixing chamber (Model 400; Altex, Berkeley, CA, U.S.A.) in conjunction with two reciprocating pumps. In the latter case, the flow-rates of the individual pumps were adjusted to maintain a constant total flow but linearly varying volume fractions at 400 regularly spaced intervals over the gradient.

Detectors

The commercial UV absorption detector (Model 153-00; Rainin, Woburn, MA, U.S.A.) and the commercial RI detector (Model R401; Waters Assoc., Milford, MA, U.S.A.) used were of standard design. To minimize the contributions of detector volume in the RI chromatograms, a dual-beam RI detector based on Fabry-Perot interferometry[3] was used. The interferometric detector[3] was modified to provide an optical pathlength of 1 cm and an internal volume of 8 μl. Data handling was accomplished by an on-line minicomputer (Model PDP 11/10 with LPS-11 laboratory interface; Digital Equipment, Maynard, MA, U.S.A.).

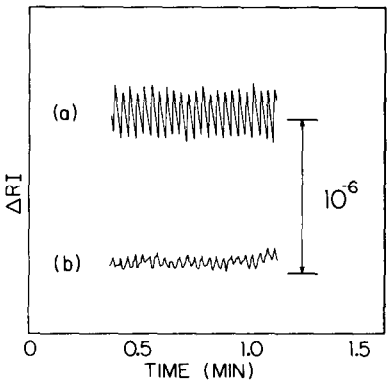
RESULTS AND DISCUSSION

The main reason for the series arrangement is to balance the sample and the reference flow cells. It is therefore important to compare the noise level here to that of a conventional arrangement. Using the arrangement in Fig. 1b and a commercial RI detector, we obtained noise level comparisons due to a reciprocating pump with

chloroform as the eluent, as shown in Fig. 2. The peak-to-peak pressure fluctuations due to the stroking of the pump are four times worse using the conventional, static reference cell, Fig. 2a, compared to the series arrangement, Fig. 2b. The residual fluctuations in the latter is probably due to a slight mismatch in the inlet tubes to the two cells, and a slight pressure drop across the delay loop. A more careful optimization of the length *vs.* diameter of the delay loop may bring further improvements. We have also investigated the arrangement in Fig. 1a. For this particular commercial detector, the inlet tubes have a much larger pressure drop than the outlet tubes, due to the particular design for temperature equilibration. The arrangement in Fig. 1a thus creates even worse a pressure difference between the two cells compared to the conventional arrangement. The noise for the former was found to be about a factor of four worse! This emphasizes the need to design the series arrangement with care. When the interferometric detector is used instead, the arrangement in Fig. 1a is found to be satisfactory for pressure equalization. Fig. 2 demonstrates that the series arrangement allows at least a factor of 4 improvement in detectability, if the main source of noise is pressure fluctuations.

The utility of the series arrangement for temperature equilibration is much more difficult to illustrate. One expects this to be dependent on the liquid flow-rate, thermal properties of the liquid and the cell body, internal surface area of the cell and tubing relative to the volume of the liquid, the total heat capacities of the liquid *vs.* the cell body and the net temperature difference. In any case, one expects thermal equilibration to be at least as good, if not better, in the series arrangement.

To demonstrate that the normal chromatographic information is retained we studied the elution of benzene in acetonitrile using the interferometric RI detector with a 88-μl delay loop. The untreated response from the detector is shown in Fig. 3a. The response looks like a derivative, but as explained earlier, it depends on the



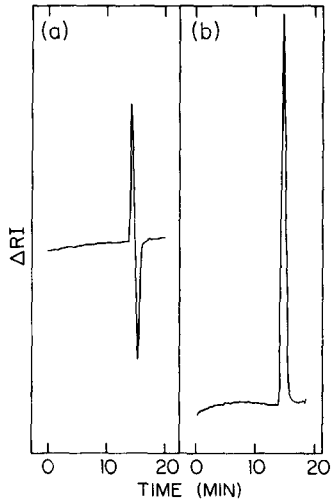
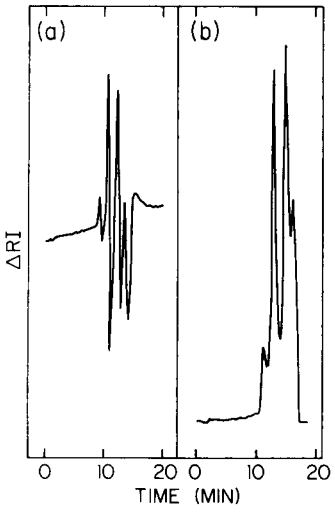


Fig. 2. Noise level due to pressure fluctuations in a conventional arrangement (a) and a series arrangement (b).

Fig. 3. RI response (a) and reconstructed chromatogram (b) of the elution of benzene in acetonitrile. Flow-rate: 0.24 ml/min. Delay: 88 μl. Full scale: 10^{-7} RI units.

chromatographic peak width and the delay volume. The gently sloping baseline is indicative of long-term temperature drifts in the detector at this very sensitive scale, but it is difficult to do a control experiment to see how much improvement was made possible by the series arrangement. Using the simple algorithm described earlier (no adjustable parameters), the chromatogram in Fig. 3b was obtained. The peak width, shape and height are as expected in a normal chromatogram. It should be noted that for a changing baseline, only the signal superimposed on the baseline should be used in correcting the response at a time V/F min later. Otherwise, the calculated chromatogram will show an incorrect baseline. We find that a linear interpolation of the detector output from the start to the end of the chromatogram is sufficient to account for the true baseline. One can see that the calculated response at any time is derived from all times $m(V/F)$ min earlier in the separation, where m includes every integer from 1 to V'/F. The resulting chromatogram will thus provide an averaging effect on any baseline noise, as long as the noise is random.

To show that this scheme works even for complex chromatograms, we studied the separation of benzene, cumene and 1,2,3,5-tetramethylbenzene using acetonitrile as the eluent. This choice of conditions results in partially resolved peaks for the three components. The untreated response is showen in Fig. 4a while the calculated response is shown in Fig. 4b. The results are again as expected, and reproduce even an impurity peak eluted before benzene. To show that this concept can be applied to the UV absorption detector as well, a similar separation is shown in Fig. 5. The relative peak heights are different from Fig. 4 because a different property is monitored. In Fig. 5a, one can even recognize the contributions of two contaminants



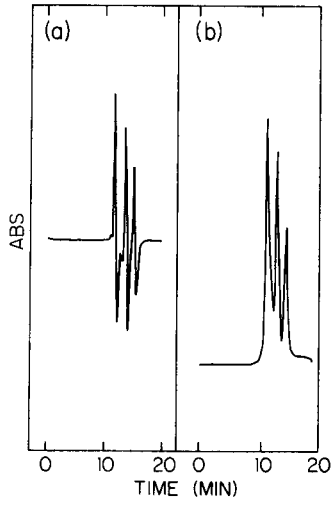


Fig. 4. RI response (a) and reconstructed chromatogram (b) of the separation of a mixture of benzene, cumene and tetramethylbenzene in acetonitrile. Flow-rate: 0.33 ml/min. Delay: 88 μl. Full scale: 10^{-7} RI units.

Fig. 5. Absorption response (a) and reconstructed chromatogram (b) of the separation of a mixture of benzene, cumene and tetramethylbenzene in acetonitrile. Flow-rate: 0.33 ml/min. Delay: 225 μl. Full-scale: 0.08 absorbance units.

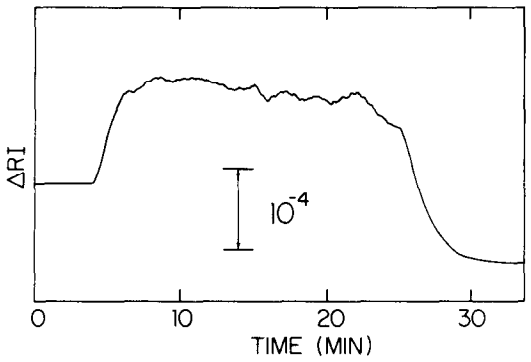
immediately before and immediately after the benzene peak, while a normal chromatogram like Fig. 5b tends to make them more obscure.

The above clearly demonstrates that the observed response must first be converted back to conventional chromatograms before any attempt at interpretation. The "heights" of the differential-looking response cannot be directly related to the concentrations of the analytes[2], since they depend on the particular signal V/F min earlier. Even for the case of a single component, the "height" changes with N (number of theoretical plates) and k' (capacity factor) for the particular separation. The "baseline" of the differential-looking response is similarly affected. So, the zero-crossing point cannot be used for determining the retention time of an analyte[2]. Differential detectors in series therefore cannot reduce interference due to unresolved chromatographic peaks.

Perhaps the worst case for balancing the sample and the reference flow cells is gradient elution in LC. A typical gradient may involve an RI change of 0.01–0.10 units. The difficulty is to try to reproduce the eluent composition at any arbitrary time at the reference cell. If a gradient is used with a linearly varying volume fraction, it can be seen that a series arrangement will produce a constant difference signal. The magnitude of this difference signal is simply the total RI change multiplied by the ratio of the delay volume and the total volume of the gradient. If desired, this constant difference can even be nulled out optically by the detector. A test of this concept using a commercial system programmed to generate a linear gradient from pure chloroform to pure carbon tetrachloride at 5% per min and at 1.0 ml/min flow is shown in Fig. 6. We note that the null point for this detector (as seen from the extreme left and the extreme right of the chromatogram) varies with the RI due to the inherent design of the optics. Taking this into account, the shape of the baseline is as predicted. In fact, the difference signal is about 1/100 of the difference in RI between chloroform and carbon tetrachloride, as predicted by a 200-μl delay loop and a 20-ml gradient. A consistent sinusoidal contribution is noticeable along the gradient, as well as some other irregular components. These were determined to be due to the inability of the programmer to generate a truly linear gradient, because of the switching of the valves and incomplete mixing. Naturally, these contributions to the baseline will be magnified in the conventional arrangement. The use of series detection therefore provides a constant background throughout the gradient to facilitate monitoring of the separation.

It should be noted that a constant baseline will not be obtained during a linear gradient run if the components of the eluent do not form an ideal solution. Such is the case for water and acetonitrile, for which a gradient run is shown in Fig. 7. The volume change causes a varying difference in the two flow cells, which is recorded in Fig. 7. In principle, one can use a non-linear program to generate a gradient to maintain a constant difference in the two cells as before, but it is easier to stay with ideal solutions.

In summary, we have shown that using differential detectors in series can be advantageous in chromatography because a better approximation to true balance can be achieved. The concept is demonstrated here for RI and absorption measurements in LC, but should be also applicable to other differential measurements, such as thermal conductivity detection in gas chromatography.



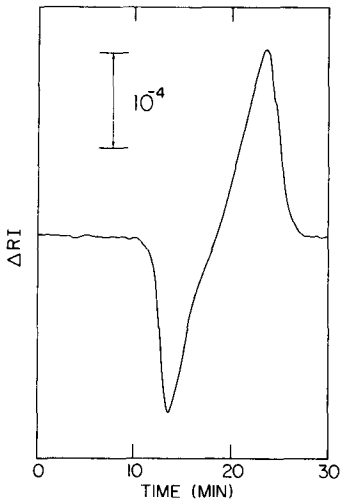


Fig. 6. Baseline response of a linear gradient from chloroform to carbontetrachloride at 5% per min. Flow-rate: 1.0 ml/min. Delay: 200 μl.

Fig. 7. Baseline response of a linear gradient from water to acetonitrile at 5% per min. Flow-rate: 1.0 ml/min. Delay: 400 μl.

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