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Influence of leaks in the liquid chromatographic instrument on analytical results

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Abstract

Leaks in a liquid chromatographic instrument can have a markedly detrimental effect on accuracy or precision, depending on circumstances, of the analytical result. The effects which can be observed depend on which area within the instrument is leaky. A leak between pump and injector usually increases retention times, theoretical plate numbers, peak areas and peak heights, depending on the van Deemter curve. A leak between injector and column increases retention times and plate numbers, decreases heights but does not influence peak areas. A leak between column and detector has little observable effects, such as a broadening of very narrow peaks, and a negligible increase of the retention times. All these features are explained theoretically and documented with the separation of a four-component mixture on a C_{18} reversed-phase column.

Keywords: Leaks; Quantitative analysis

1. Introduction

Leaks within an instrument for high-performance liquid chromatography (HPLC) or a system for low-pressure column liquid chromatography (CLC) are not uncommon and are well-known to every person who works with these methods. Usually they are regarded as inconvenient and unwanted but there is no general awareness of their detrimental effects (besides crystallisation and corrosion in the case of buffers). Leaks can influence retention times, peak widths and thus theoretical plate numbers, peak areas and peak heights, i.e., all parameters which are used for qualitative or quantitative analysis. As a consequence the analytical result is affected.

However, predicting the effects which are caused by a leak is not a straightforward process. There is no complex mathematics involved but probably most users of HPLC or CLC are not aware of the underlying theory. Therefore it may be surprising at first glance that the effects depend highly upon the location within the chromatographic system where the leak occurs. As a general rule, a leak has more consequences the larger it is (this is obvious) and the further upstream in the apparatus it is located. Compact HPLC systems are much less convenient for the detection of leaks whereas modular instruments are easier for the personnel to monitor.

Regarding the possible consequences it is surprising that leaks do not seem to be a topic discussed in the literature on column liquid chromatography.

2. Theory

It is necessary to distinguish three possible locations within the instrument where a leak can occur: between pump and injector, between injector and

column, or between column and detector. The effects on pressure drop Δp, retention time t_R, theoretical plate number N, peak height h_p and peak area A_p are discussed here. A symbol without a prime represents the original value without leak whereas a prime, e.g., $\Delta p'$, represents the affected value.

2.1. Leak between pump and injector

A leak before the injector has exactly the same consequences as a pump failure which results in a lower flow-rate through the column and all other parts of the apparatus. The effects are governed by the van Deemter curve of the column, i.e., by the way peak broadening is influenced by a variation of the volume flow-rate.

The influences on pressure drop and retention times are straightforward; the first is decreased whereas the latter is increased proportional to the decrease of the volume flow-rate F of the mobile phase through the column:

$$\Delta p' = \Delta p \frac{F'}{F} \qquad (1)$$

(It is assumed that the pressure drop through capillaries and all other parts of the instrument is negligibly small.)

$$t'_R = t_R \frac{F}{F'} \qquad (2)$$

The theoretical plate number depends on the van Deemter curve which describes the relation between the height of a theoretical plate H and the linear flow-rate u. This cannot be stated in general but is typical for every column. However, it is a fact that most liquid chromatographic columns are used well above their van Deemter optimum; this allows shorter separation times whereas for a well-packed column the loss of separation performance (or theoretical plates) is not too severe. For both theoretical and experimental data given in this paper the results are predicted and obtained, respectively, based on the fact that H increases linearly with decreasing u when the column is operated above the van Deemter optimum. For convenience these parameters are described and measured in converted form, namely as theoretical plate number N ($N = L_c / H$, with $L_c =$

column length) and as volume flow-rate F ($F =$ const $\cdot u \cdot d_c^2$, with $d_c =$ column inner diameter). In this form the "van Deemter curve" has a maximum of N and, in the flow-rate range where the column is used here, N increases linearly with decreasing F; the relationship between the two terms is described by the slope S (see the N/F curve given in Section 3). Under these circumstances the theoretical plate number observed under leaky conditions N' is described by the equation:

$$N' = N - S(F - F') \qquad (3)$$

For concentration sensitive detectors such as the UV, fluorescence, or refractive index detector, peak height is nothing more than the expression of peak maximum concentration c_{max} which is described by [1]:

$$c_{max} = \frac{m_i}{V_R} \sqrt{\frac{N}{2\pi}} = \text{const} \frac{m_i}{V_R} \sqrt{N} \qquad (4)$$

with $m_i =$ injected mass of solute ($=$ injected volume $\times$ concentration) and $V_R =$ retention volume. Since m_i and V_R (measured at the column end or at the detector output) are not influenced by a leak before the injector, c_{max} and thus h_p only depend on N giving the following relationship for h'_p:

$$h'_p = h_p \sqrt{\frac{N'}{N}} \qquad (5)$$

Peak area can be described starting with the definition of N:

$$N = 2\pi \left(\frac{h_p t_R}{A_p} \right)^2 \qquad (6)$$

which gives:

$$A_p = h_p t_R \sqrt{\frac{2\pi}{N}} \qquad (7)$$

and leads, with the use of Eqs. (5,2), to:

$$A'_p = h'_p t'_R \sqrt{\frac{2\pi}{N'}} \qquad (8a)$$

$$A'_p = A_p \frac{F}{F'} \qquad (8b)$$

Summary of Eqs. (1)–(8b): a leak before the injector decreases the pressure drop but increases

retention times, plate numbers, peak heights and peak areas if the flow-rate with leak F' is still above the van Deemter optimum of the column.

2.2. Leak between injector and column

A leak before the column leads to the same consequences as a leak before the injector but in addition only a part of the injected sample m_i reaches the column. (It is assumed that the mixture of sample and eluent is homogeneous over the cross-section of a capillary or fitting at the place where the leak occurs.) m_i' is smaller than m_i according to the degree of the leak:

$$m_i' = m_i \frac{F'}{F} \qquad (9)$$

Pressure drop $\Delta p'$ and retention times t_R' can be described according to Eqs. (1,2). Again, it is assumed that the pressure drop in capillaries etc., is small compared to the drop over the column. In addition, the theoretical plate number N' follows Eq. (3). Δp, t_R and N are not influenced by sample mass as long as column overload is avoided.

Peak height, however, is affected by the loss of sample. Eq. (4) can be re-written as:

$$h_p = \mathrm{const}' \frac{m_i}{V_R} \sqrt{N} \qquad (4a)$$

The retention volume is not influenced by this type of leak but for m_i' Eq. (9) needs to be considered. This gives:

$$h_p' = h_p \frac{F'}{F} \sqrt{\frac{N'}{N}} \qquad (10)$$

The effect of F'/F overrides the one caused by the fact that N' is larger than N and peak height decreases.

For peak area, the combination of Eqs. (8a), (10), (2) gives the (maybe surprising) result:

$$A_p' = A_p \qquad (11)$$

Peak area is unaffected because the area increase by a lower flow-rate is exactly compensated by the area decrease due to sample loss.

Summary of Eqs. (9–11) and the preceding discussions: a leak between injector and column de-

creases pressure drop and peak heights, increases retention times and plate numbers, and does not influence peak areas if the flow-rate with leak F' is still above the van Deemter optimum of the column.

2.3. Leak between column and detector

In this case the separation on the column remains unaffected but the eluate is split before it enters the detector. For concentration sensitive detectors this means that the chromatogram is not influenced at all with the possible exception of retention times and the shape of very narrow peaks.

The pressure drop remains constant if we assume again that the flow resistance by the connecting capillaries and the detector cell is low. Retention times could increase very slightly if the volume between the location of the leak and the cell is not small enough. This additional retention time Δt_{det} is defined as:

$$\Delta t_{det} = t_{det}' - t_{det} = \frac{V_{det}}{F'} - \frac{V_{det}}{F} = V_{det} \frac{F - F'}{FF'} \qquad (12)$$

with V_{det} not being the nominal detector cell volume as given in the instrument manual but the volume of capillaries, heat exchangers, fittings etc., between the leak and the cell entrance. This should not be more than a few microliters which gives rise to a very small Δt_{det}. (A pessimistic example: if $V_{det} = 100$ μl, a value being too high for a well-designed HPLC instrument, $F = 1$ ml/min and $F' = 0.5$ ml/min representing a 50% leak, $\Delta t_{det} = 0.1$ min = 6 s.) Usually V_{det} is not known.

A detector should be designed in such a way that it does not lead to additional broadening of the peaks. The maximum allowed volume of the detector cell, V_{cell}, is defined as [2]:

$$V_{cell} = \frac{\theta V_R}{\sqrt{N}} \qquad (13)$$

with $\theta =$ fraction of peak broadening ($\theta = 0.01$ gives 1% broadening etc.). Unfortunately a standard 8 μl cell can be too large even for early eluted peaks from a 25 cm×4.6 mm column [3]. Concerning a leak between column and detector we need to consider that now the peak volume, as registered by the detector, is lower than the peak volume at the column outlet. This makes early eluted peaks prone

to broadening, i.e., to a decrease of theoretical plate numbers and heights whereas peak areas should not be affected.

For peaks broad enough not to be affected by this mechanism the leak has no influence on their plate number, height or area. A possible exception, however, is the plate number due to the slightly longer retention time. N is most often calculated as:

$$N = 16\left(\frac{t_R}{w}\right)^2 \tag{14}$$

with w = peak width at the base. w is not influenced by the leak because, although the flow-rate through the detector cell is lower, the volume which needs to pass the cell is also lower:

$$w = \frac{V_{peak}}{F} = \frac{V'_{peak}}{F'} \tag{15}$$

By combination of Eq. (14) and the expression for the slightly increased retention time, $t_R + \Delta t_{det}$, one obtains:

$$N' = N\left(\frac{t_R + \Delta t_{det}}{t_R}\right)^2 \tag{16}$$

which means that in principle the number of theoretical plates increases.

Peak heights are not influenced (if no peak broadening occurs) because the usual liquid chromatographic detectors measure concentration not mass. A leak does not alter the concentration of the eluate. And, if peak widths (Eq. (15)) and heights are not affected, peak areas also remain constant.

Summary: a leak between column and detector does not influence the chromatogram with the exception of very narrow peaks which are broadened and thus decreased in their height. A slight increase in retention time could be observed if the volume between the leak site and the detector entrance is relatively high.

3. Experimental

Conditions
Column: 7.5 cm×4.6 mm
Stationary phase: Zorbax SB C_{18}, 3.5 μm (Rock-

land Technologies, Newport, DE, USA)
Mobile phase: water–ethyl alcohol (7:3, v/v)
Volume flow-rate: 2 ml/min (because this mobile phase is rather viscous, a pressure drop Δp of ca. 300 bar was obtained)
Detector: Jasco UV-975 (Jasco Corp., Tokyo, Japan), 268 nm
Data evaluation: GynkoSoft Chromatography Data System, Version 5.32 (Gynkotek, Germering, Germany)

Because leaks are not convenient this study was performed with a non-corrosive and non-toxic mobile phase without salt additives. No additional parts such as T-pieces were built into the flow path because this might give rise to extra band broadening or unpredictable flow effects. The leaks were obtained by loosening the appropriate fittings. Since it is impossible to loosen a fitting in a well-defined, "quantitative" manner, it was not tried to obtain specified leak rates but the data points are arbitrary. Leaks are given in % of the original volume flow-rate:

$$Leak = 100\frac{F - F'}{F} \ [\%] \tag{17}$$

Example: a 10% leak means that still 90% of the total eluent is flowing through the chromatographic system. F was determined immediately before a series of different leaks were generated and again at the end of a series of experiments; F and the various F' were measured at the detector outlet with a 10 ml volumetric flask and a stop-watch. F' was always kept above the van Deemter optimum, i.e., within the linear part of the curve.

The test mixture consisted of a non-retained compound, thiourea (2 mg/ml), a poorly resolved peak pair, veratrole and acetophenone (1 mg/ml each) and a compound eluting at ca. $k' = 6$, nitrobenzene (0.25 mg/ml). The latter was used for the determination of retention times, peak heights and peak areas. The mixture was dissolved in the mobile phase. Sample volumes of 25 μl were injected into a Rheodyne 7413 valve with an internal 5-μl loop (Rheodyne, Berkeley, CA, USA).

4. Results and discussion

The modified van Deemter curve, in the form of $N = f(F)$, was determined for nitrobenzene between 0.1 and 1.9 ml/min. It is shown in Fig. 1. The almost linear part between 0.8 and 1.9 ml/min has a slope S of -2180 min/ml. This value was used in Eq. (3) to calculate the theoretically expected N' data which then were again used in Eqs. (5,10).

Fig. 2 presents the results for the nitrobenzene peak obtained with a leak between pump and injector. Note that the three diagrams for retention time, peak height and peak area all have two x axes, the upper one giving the flow-rate F or F' measured at the detector outlet and the lower one showing the corresponding leak according to Eq. (17). y axes do not begin at zero. White squares represent the theoretically expected data according to Eqs. (2,5) and Eqs. (8b). Theoretical and experimental data (black dots) match well. A chromatogram of the undisturbed separation and of the largest investigated leak, here 46%, is shown in the fourth quadrant. Besides the expected increase of times, heights and areas an improved resolution of veratrole and acetophenone can also be observed due to the increased number of theoretical plates at ca. 1 ml/min.

Fig. 3 shows the analogue for leaks between injector and column. Theoretical data were calcu-

Fig. 2. The influence of a leak between pump and injector on retention time, peak height and peak area of the nitrobenzene (last) peak and on the chromatogram. ● = experimental data points, □ = theoretically calculated data points.

lated according to Eqs. (2,10,11). Again theory and experiment show good accord and the resolution of the critical peak pair improved.

Fig. 4 shows experimental data only because the values for the rather late eluted nitrobenzene peak should not be affected at all by a leak after the column. A possible increase of the retention time according to Eq. (12) cannot be predicted because V_{det} is not known. The observed slight increase of the peak height cannot be explained and is probably an experimental artifact. Retention time and peak area behave as expected and remain unchanged. The chromatograms show that at the 47% leak the height of the thiourea (first) peak is smaller than the height of the nitrobenzene (last) peak because it undergoes band broadening in the detector cell as discussed before. Without disturbance the thiourea peak is higher than the nitrobenzene peak at the given sample composition and detection wavelength. The
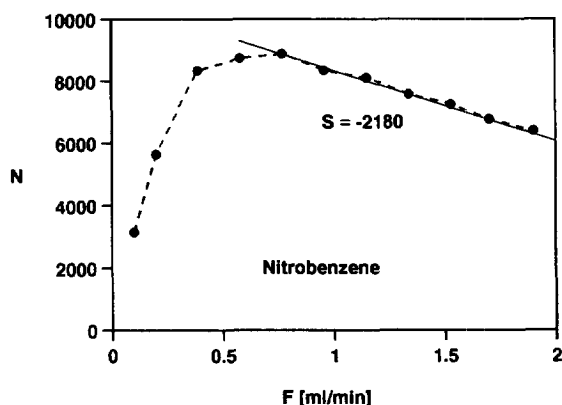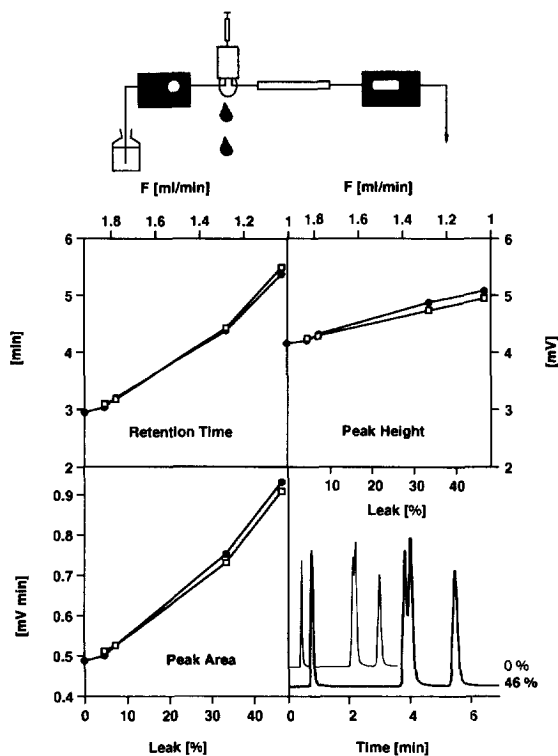

Fig. 1. The influence of volume flow-rate on the plate number of the nitrobenzene peak in water–ethyl alcohol, 7:3 (a modified van Deemter curve). The part between 0.8 and 1.9 ml/min can be described by a straight line of slope -2180 min/ml. For experimental conditions see Section 3.
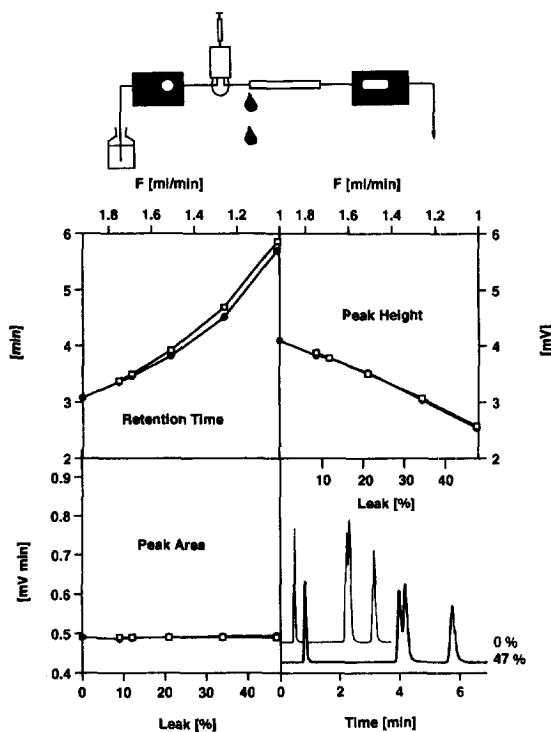
Fig. 3. The influence of a leak between injector and column on retention time, peak height and peak area of the nitrobenzene (last) peak and on the chromatogram. ● = experimental data points, □ = theoretically calculated data points.
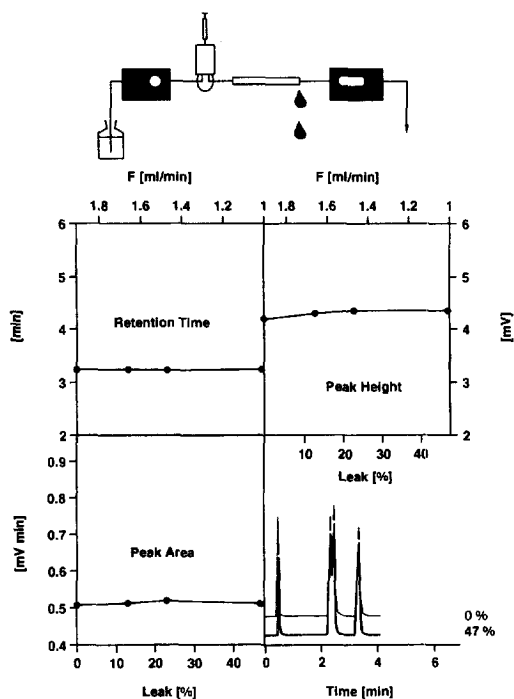

Fig. 4. The influence of a leak between column and detector on retention time, peak height and peak area of the nitrobenzene (last) peak and on the chromatogram. Only experimental data points are shown because this type of leak in theory has no influence on retention time, peak area or peak height if the peak is wide enough not to be broadened by the detector cell.

resolution of veratrole and acetophenone is not influenced.

5. Leaks and quantitative liquid chromatographic analysis

The influence of a leak on the parameters of interest in analytical liquid chromatography, retention time, peak height and peak area, is the larger the more upstream it occurs. At the end of the flow path, after the detector, it has no influence at all and effects are very minor if the leak is located between column and detector. With a leak between injector and column at least peak areas remain undisturbed. If the leak occurs before the injector, however, all three parameters are strongly influenced. In the case presented here the separation was even improved by such a leak because the numbers of theoretical plates

increased which resulted in a better resolution (the negative effect is the increase in retention times). This of course does not mean that a leak before the injector is in any way advantageous; it only observes that in the vast majority of cases a liquid chromatographic separation is performed above its van Deemter optimum and that a decrease of the flow-rate improves the resolution.

Does a leak affect accuracy or precision? Of course there is no detrimental effect on quantitative analysis if the leak is constant and "undisturbed" during the whole time of an assay from the determination of the calibration curve to the completion of the analyses of all samples whose content is calculated based on this curve (the sometimes disastrous corrosive consequences on the instrument are not discussed here). If leaks occur sporadically and are fixed soon this will affect precision. If leaks occur at a certain time point during the course of a

full analysis cycle (calibration plus determination) and are not fixed this will affect accuracy. In any case, if a leak is detected during analytical work the affected data need to be rejected; with the exception of cases where the time point of the leak can be reconstructed without doubt it is necessary to repeat the full analysis cycle.

Can a leak be detected by the instrument software itself? This of course would be highly desirable for the performance of system suitability tests and validation. The simplest way to do this would be a flow control and comparison at the pump outlet and the detector outlet; although this would not give a hint on the location of a leak it could be used for the activation of an alarm and/of a pump stop. A diagnosis from the chromatogram itself is only possible in the case of a leak between injector and column: this leads to an increase of retention times and (depending on the van Deemter curve!) a decrease of peak heights whereas peak areas remain constant. Such a combination of features cannot be generated by a single other event. However, it is not possible to distinguish from the chromatogram (and from the pressure drop) between a decrease of the pump discharge and a leak between pump and injector because both give identical effects on retention times, peak heights and areas, and since a leak between column and detector has almost no effects it is hopeless to try to detect it from the chromatogram.

As a conclusion it can be stated that leaks are not only irksome but that they must be strictly avoided because they can have severe influences on analytical precision or accuracy.

Acknowledgments

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