

Chapter 4

Application of Solid Scintillators in High Pressure Radio Column Chromatography

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INTRODUCTION

Column chromatography of compounds labelled with a weak β -emitter is applied in two different ways. The most common method is the discontinuous measurement of the radioactivity in the individual samples from a fraction collector. The samples are counted with the appropriate scintillators and solvents in a non-aqueous environment. On the other hand, continuous measurement of radioactivity in the effluent of a chromatographic column by means of a flow cell may be applied. Before enumerating the advantages and disadvantages of both systems, it is better to carry out some calculations in order to get an objective insight into the theoretical possibilities of both detecting systems.

Table 1 shows the results of the discontinuous counting of 10 nC. The efficiency is supposed to be 80%, the background 100 c.p.m. One minute counting time produces a resulting signal of 20 000 counts. The signal to noise ratio is 2000.

Table 2 applies to the continuous measurement of the same amount of radioactivity. Let the efficiency be 50%. An eluent flow rate of 1 ml/min and a cell volume of 0.1 ml results in 0.1 min residence time of each particle in the detector. So the counting time of the radioactivity is 0.1 min. The signal amounts to 1110 counts. The elution time of the total amount of radioactivity is 5 min, so the background contribution becomes 500 counts and the signal to noise ratio is decreased to 50. This means a decrease by a factor of 40 in comparison with the discontinuous measurement. Moreover one should remember that the signal to noise ratio cannot be improved in the case of the continuous measurement unless the counting time is increased. This can only be achieved by a decrease in the flow rate, which is in contradiction with the demand for a rapid separation of the compounds under investigation. On the other hand, the signal to noise ratio in the case of discontinuous measurement can be improved very simply by the choice of a longer counting time of the individual samples. This can be done without violating the separating conditions.

From the foregoing example it is quite clear that flow counting can never compete with discontinuous measurement. Consequently it may be wondered why continuous measurement is applied. The reasons for this are merely practical and are not difficult

Table 1. Discontinuous measurement.

Radioactivity	10 nC
Efficiency	80%
Counting time	1 min
Signal	~ 20000 counts
Background	100 c.p.m.
Noise = $\sqrt{\text{Background}}$	10 c.p.m.
Signal to noise ratio	20000/10 = 2000

Table 2. Continuous measurement.

Radioactivity	10 nC
Efficiency	50%
Flow rate (F)	1 ml/min
Cell volume (V)	0.1 ml
Counting time (V/F)	0.1 min
Signal	1110 counts
Background	100 c.p.m.
Elution time	5 min
Background contribution	$5 \times 100 = 500$ counts
Noise = $\sqrt{\text{Background contribution}}$	~ 22 counts
Signal to noise ratio	1110/22 \approx 50

to understand:

1. In contrast to the discontinuous method the continuous method is non-destructive in the case of weak β -emitters.
2. The method is fast. In the case of discontinuous counting all fractions have to be examined for radioactivity. The preparation of the samples is time-consuming.
3. The method is fully automatic. The ratemeter/spectrometer signal as a function of time can be registered by means of a strip chart recorder.

Weighing the advantages and disadvantages of both methods against one another, it was decided to combine both techniques in order to profit by the accuracy of the discontinuous method as well as by the gain of time of the continuous counting.

With this end in view it is our intention to construct a chromatographic system in which the flow cell serves as a level detector and steers as such a fraction collector. In other words, as soon as the background rises to an adjustable level, the effluent is collected until the background returns to its original level. The collected fraction can then be prepared for discontinuous measurement.

CONSTRUCTION OF THE FLOW CELL

For a good operation of this combined system it is necessary to know the properties of the flow detector. We constructed a flow cell suitable for continuous measurement of weak β -radiation in conjunction with a high pressure chromatographic column. With the construction attention has been paid to the possibility of changing the scin-

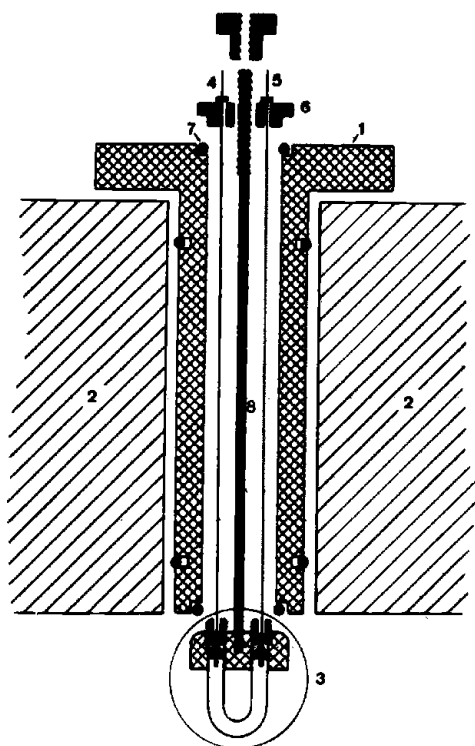


Fig. 1. Cell holder with flow cell. 1 = aluminium cylinder, 2 = photomultiplier housing, 3 = flow cell (part encircled Fig. 2), 4 and 5 = inlet and outlet capillaries, 6 = lid, 7 = 'O' ring, 8 = screw.

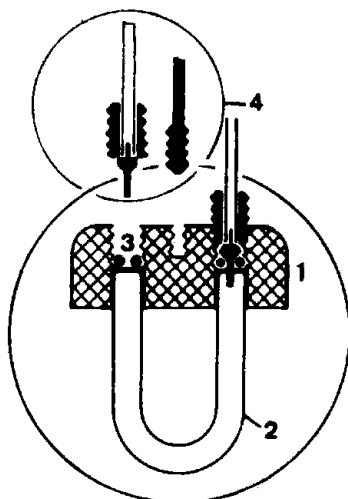


Fig. 2. Flow cell. 1 = socket, 2 = quartz U-pipe, 3 = 'O'-ring, 4 = joint and screw.

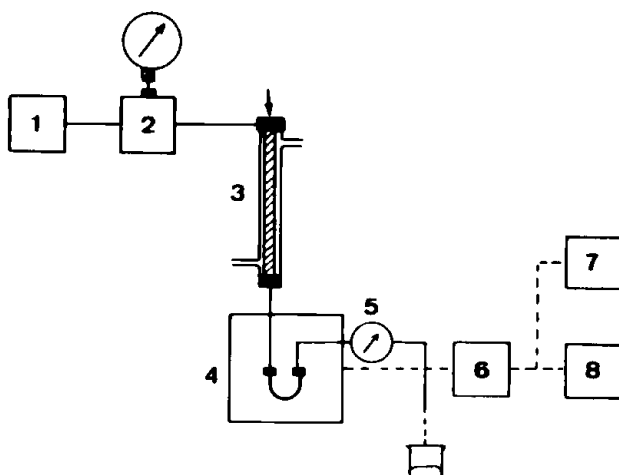


Fig. 3. Scheme of the apparatus. 1 = eluent reservoir, 2 = pump, 3 = thermostated column, 4 = detector cell mounted in counting chamber of the photomultiplier assembly, 5 = flow meter, 6 = ratemeter/spectrometer, 7 = multi-channel recorder 8 = recorder.

tiltting material, thus enabling a free choice of scintillator and a rapid renewal in case of contamination.

Figure 1 shows the cell holder and cell constructed in our laboratory. The cell holder consists of a hollow cylinder. The actual cell is a quartz U-pipe mounted on a socket which is attached to the lower part of the holder.

Figure 2 shows the actual cell and the joints for the inlet and outlet tubing. The cell is filled with solid scintillating material. Several commercially available scintillators were used.

Figure 3 shows the chromatographic and electronic system. The eluent is pumped through by a pulsating pump. The pump and the injection port are connected by a teflon tube. The connector at the bottom of the column is joined to the detector cell in the same way. The scintillations caused by the interaction of the radioactivity and the scintillating material are detected by the photomultiplier assembly. The recorder signal of the ratemeter/spectrometer is fed into a strip chart recorder. The digital output signal is fed into a multi-channel analyser used as a multi-scaler.

EXPERIMENTS

Relative static efficiencies

By measuring the relative static efficiency, the scintillator with the best properties was chosen for further experiments. The results are shown in Table 3. It is obvious that PPO and PTP are the most efficient scintillators. However, POPOP was preferred for further experiments because it shows better cell packing properties and maintains a constant flow rate. The flow cell filled with POPOP possesses an effective fluid volume of 0.1 ml.

Table 3. Relative static efficiency of several scintillators for ^{14}C -labelled ethanol.

Scintillator	Relative static efficiency (S_{rel}) (%)
PPO	100
PTP	78.6
POPOP	54.3
DPS	46.7
<i>trans</i> -Stilbene	43.5
Anthracene	34.9
ANPO	34.5
LiCl	24.6
Naphthalene	17.0

Table 4. Absolute static efficiency of POPOP for ^{14}C -labelled amino acids and HTO.

Compound	Absolute static efficiency (S_{abs}) (%)
Aspartic acid	50.9
Threonine	53.4
Glutamic acid	53.2
Serine	50.7
Proline	53.6
HTO	5.4

Absolute static efficiencies

The results of the determination of the absolute efficiency of POPOP for some ^{14}C -labelled amino acids and tritiated water are shown in Table 4. The results are in good agreement with one another. The high efficiency values found are the more remarkable in view of the small effective volume of the detector.

Calibration curve

For quantitative measurements a calibration curve has to be realized. Figure 4 shows the calibration curves for carbon-14 and tritium radioactivity. The input signal is represented by the amount of radioactivity injected, the output signal by the number of counts recorded on the multi-channel analyser.

The output signal of the detector as a function of the input signal shows good linearity. This figure also shows the dependence of the output signal on the β -radiation energy as can be concluded from the different slopes of the calibration curves for carbon-14 and tritium.

Peak broadening

The most important characteristic of a flow cell is its peak broadening effect at

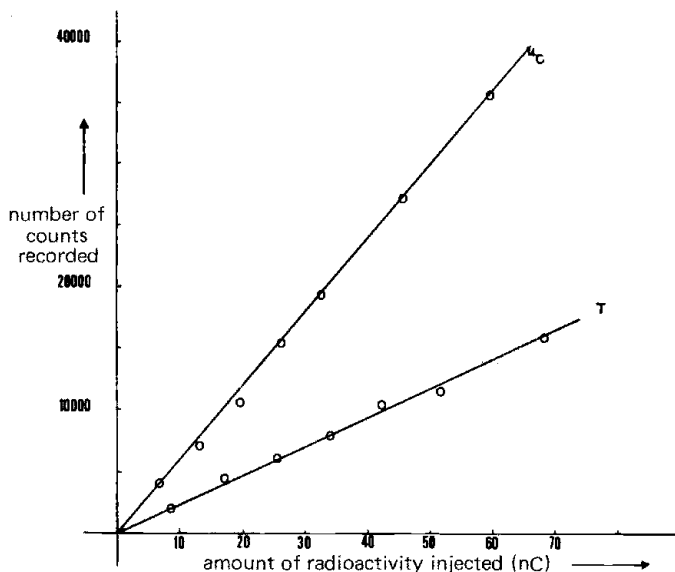


Fig. 4. Calibration curve for ^{14}C -labelled aspartic acid and for ^3H -labelled water.

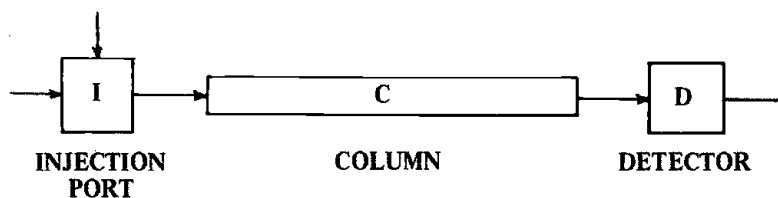


Fig. 5. Chromatographic system.

a certain flow rate. The detector is expected to deliver a signal time function which is a true representation of the elution function.

Figure 5 shows the chromatographic system: the injection port, the feed line to the column, the column, the feed line to the detector and the detector itself. The signal time function of a component, the elution function, has a Gaussian shape and can be characterized by the standard deviation. A concentration peak leaving the injection port will disperse further in the feed line to the column and subsequently in the column, the feed line to the detector and the detector. These peak broadening effects caused by the individual components of the chromatographic system are independent of one another. Consequently the total dispersion can be expressed in terms of variances and summarized as shown in Fig. 6.

As mentioned above the detector is expected to deliver a true representation of the elution function. That means that the contribution of the detector to the overall peak broadening should be small. The determination of the detector contribution is

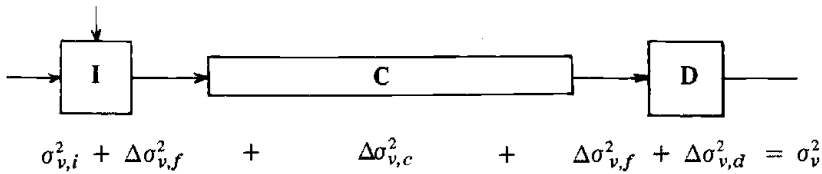


Fig. 6. Peak broadening contributions in the chromatographic system expressed in terms of variances.

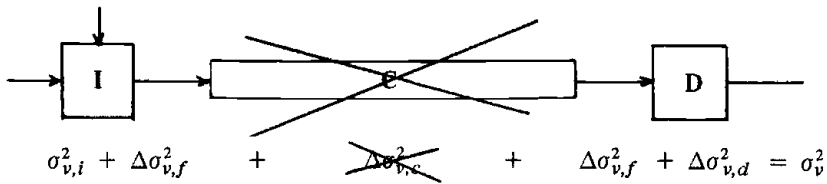


Fig. 7. Measurement of the peak broadening effect of the flow cell.

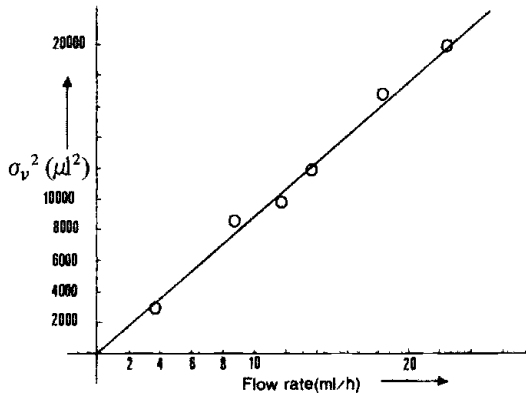


Fig. 8. Peak broadening caused by the flow cell as function of the flow rate.

possible by experimenting without a column. This is made clear in Fig. 7. The radioactivity is injected directly into the detector. By measuring the peak broadening in this way we can calculate the peak broadening effect of the flow cell, since the contributions of the other part of the system are constant.

Figure 8 shows the results. The relationship between the flow rate and the variance is an important property of the flow cell. Experiments including a column provide the overall peak broadening. Consequently one can decide on an acceptable contribution of the flow rate to the overall peak broadening.

Contamination

In view of the peak broadening effect, another aspect has to be considered – namely the contamination by radioactive material. A longer residence time of the activity in the detector and consequently an increase of the peak broadening can be expected in cases of contamination.

In our experiments tritium-labelled water did not contaminate the scintillator at all. However, the amino acids contaminate POPOP to a high degree in the absence of a decontaminator in the eluent. The output signals have not the normal Gaussian shapes but show tailing effects. The presence of a detergent in the eluent, however, eliminates the contamination and consequently a constant background was measured before and after the passing of an amount of radioactivity through the detector.

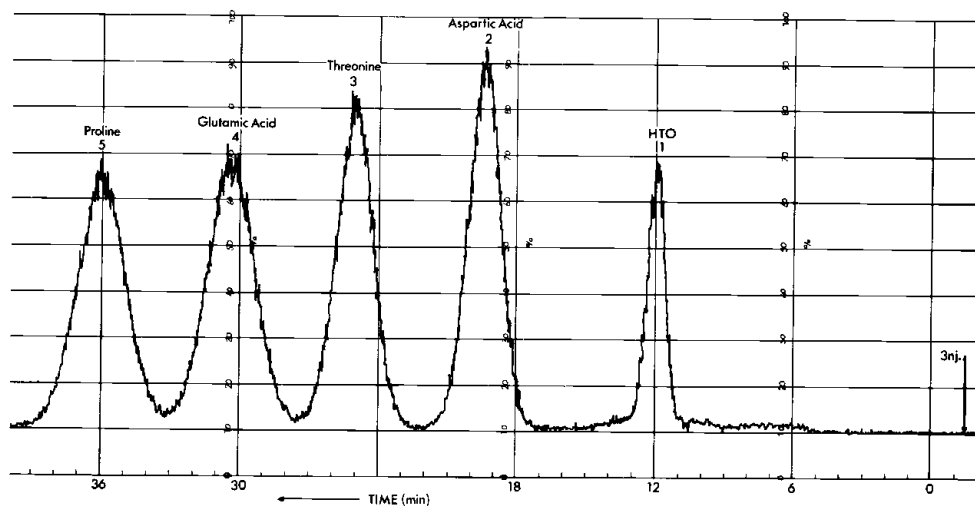


Fig. 9. Chromatogram of a mixture of (1) tritiated water, (2) aspartic acid, (3) threonine, (4) glutamic acid and (5) proline. Separation on Aminex Q-150S with citrate buffer. Counting range 25 kcpm; time constant 1.5 s; flow rate 12.4 ml/h; recorder full scale 10 mV; chart speed 1 cm/min; temperature 60°C; column dimension 50 X 0.5 cm.

Separation of a mixture of amino acids

In practice the monitor is used in conjunction with a column filled with cation exchanger. A mixture of ^{14}C -labelled aspartic acid, threonine, glutamic acid, proline and tritiated water was used as the separation example. The result of the separation is shown in Fig. 9. At first sight one may be satisfied. The separation is completed within 40 min and the components of the mixture are separated almost entirely. However, one should also remember the contribution of the flow cell to the overall peak broadening. At a flow rate of 12.5 ml/h the contribution of the flow cell to the peak broadening is 100 μl . The function of tritiated water in the mixture is now clear. Assuming interaction between ion exchanger and water, the column will disperse the tritiated water peak to a very small extent. The quantity of peak broadening of more than 100 μl will be caused by mixing in the column. In the same way every peak in the chromatogram possesses a

Table 5. Detector contribution to total peak broadening.

Compound	σ_v (μ l)	Detector contribution (μ l)		Detector contribution (%)
HTO	105	100	=	95
Aspartic acid	210	100	=	48
Threonine	235	100	=	43
Glutamic acid	260	100	=	38
Proline	287	100	=	35

detector peak broadening contribution of 100 μ l. Table 5 shows the results of these simple calculations. One can see that 95% of the dispersion of the tritiated water peak is caused by the detector cell. So only 5% is due to mixing. The detector peak broadening effect on the second component is also large, namely 48%, and decreases in the elution sequence down to proline (35%).

CONCLUSION

It is obvious that the result is affected to a great extent by the detector. Satisfaction is changed to dissatisfaction when one considers the applicability of the detector. One can assume two things:

1. The column is too efficient for the detector, so the separating system will have to be adjusted.
2. The detector is insufficient and will have to be corrected.

The first recommendation is in flagrant contradiction to the chromatographic demands. The detector should not ruin the separation. It is clear that the flow cell will have to be adjusted. This can be done by reducing the detector volume. However, this means a reduction of the signal since the counting time is proportional to the cell volume. Likewise a reduction of the efficiency means an increase of the detection limit. A solution to this difficulty is the so-called peak tracer method. If the amount of radioactive-labelled compound in a mixture is too small for flow detection one can inject a large amount of this particular compound before the actual separation of the mixture. The flow detection then indicates the place in the chromatogram for the compound under investigation. Next the separation of the mixture is carried out under the same conditions. The effluent is collected at the place in the chromatogram indicated by the peak tracer method and counted in the discontinuous way until a significant signal is recorded.

One can conclude that the combination of a flow cell, as a level detector or a peak tracer detector, with a fraction collector provides an improvement in the sensitivity of the determination and decreases the time of analysis.

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DISCUSSION

B. E. Gordon: I don't clearly understand the peak tracer method.

G. B. Sieswerda: Since the intensity of signal is dependent on the cell volume and the flow rate, some small amounts of radioactivity may give no significant signal under certain separation conditions. The only possibility for detection then is to use the peak tracer method. In this method, a much larger and measurable amount of radioactivity is injected under the same separating conditions and in this way the retention time of this particular compound is determined. During the second run, in which the unknown mixture is separated, the effluent is collected at the retention time indicated by the tracer, and counted by the more conventional discontinuous liquid scintillation counting procedure.

J. Murray: Which detergent did you use for reducing contamination of the scintillator by ^{14}C -labelled amino acids?

G. B. Sieswerda: We used a polyethylene lauryl ether (Bry-35) which is also a component of the eluent necessary for the chromatographic separation.

D. Schram: How were the several solid scintillators calibrated for their mesh size, prior to the determination of their relative efficiencies?

G. B. Sieswerda: We did not calibrate for mesh size, we just used the available products. However, depending on the chromatographic requirements, it may be necessary to recrystallize and sieve the scintillators.

B. W. Fox: Do I understand that the only reason for using POPOP as against PPO is because of its better packing properties?

G. B. Sieswerda: Yes.

B. W. Fox: Have you looked into the possibility of altering the crystal habit of PPO to utilize its much greater efficiency?

G. B. Sieswerda: No, we have not done this.

P. Johnson: Dr. Sieswerda, can I ask if you have used any mass detector in addition to your flow detector for radioactivity? The reason I ask is that one of the well accepted advantages of using a flow detector system for radioactivity in parallel with a mass detector, rather than a discrete off-line counting method, is that with discontinuous detection of radioactivity it is possible to miss peaks of high specific activity but insufficient mass to activate the mass detector. It would therefore be of interest to use your system for instance, on-line to one of the commercially available ultraviolet or other mass detector systems.

G. B. Sieswerda: This would be of interest but we have not yet used any detector other than the radioactivity flow cell.

B. Scales: High pressure liquid chromatography is often carried out using mixed solvents, and gradient elution techniques. When organic solvents are incorporated into the system, the increased solubility of the scintillator can be a serious problem. Have you considered the use of plastic scintillators in these situations, or are the counting efficiencies too low?

G. B. Sieswerda: Yes, plastic or glass scintillators can be used to overcome the problems of dissolution of conventional organic scintillators but the counting efficiencies are

unacceptably low for weak β -emitters.

P. Stanley: Have you attempted to flow-count macromolecules of biological interest, for instance proteins or nucleic acids? Does PPO affect the biological activities of these macromolecules?

G. B. Sieswerda: The answer to the first question is no. I did not use the flow cell for the detection of macromolecules. In this respect, however, one can expect contamination of the scintillating material. Macromolecules, like proteins and nucleic acids, can complex with the scintillator and can be retained on its surface. So, in answer to your second question, I must say that there is a risk of affecting the biological activities of these macromolecules.