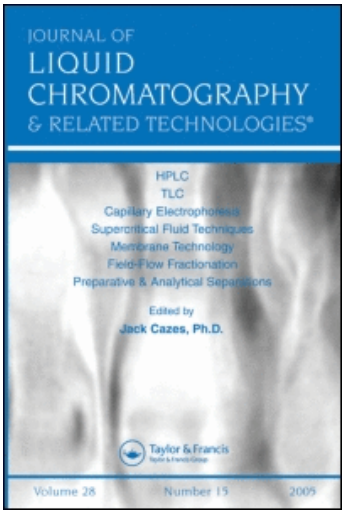

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β-PARTICLE DETECTION IN HPLC BY FLOW-THROUGH MONITORING VS. LIQUID SCINTILLATION COUNTING

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ABSTRACT

Statistical considerations suggest that fraction collection followed by liquid scintillation counting offers the possibility of greater sensitivity and accuracy for low-activity measurement of radioactivity in HPLC eluates than does continuous-flow measurement, albeit with considerable inconvenience. However, when the randomness associated with fraction collection is taken into account, presumed advantages of fraction collection largely or wholly disappear and the trend to replace fraction collection with continuous measurement is justified.

INTRODUCTION

Measurement with the flow-through monitor has displaced fraction collection followed by discrete sample liquid scintillation counting as the method of choice for profiling the elution of beta-activity from HPLC columns. But, an objective observer would only attribute such preference to the combination of convenience -- results in real-time, automatic peak integration, the coupling of radioactivity and UV traces -- with economy and, for some, an advantageous waste disposal situation. Not much concern has been given to performance which is, therefore, the subject presently addressed.

As used here, "continuous-flow" implies continuous passage of column eluate through a coincidence type liquid scintillation detector; three distinct methods of photon production -- flow over insoluble scintillator, admixing with scintillator solution, or for very energetic isotopes, Cerenkov counting -- are commonly practiced; in any case, mathematical treatment of the counting results follows the same course. On the other hand, when the term "liquid scintillation counter" is used, it implies the measurement of discrete static samples in individual containers, always with liquid scintillator present.

The equipment for both applications, especially insofar as the actual detection and measurement components and their configuration, is similar. Two photomultipliers view the sample whether it is contained in a vial for liquid scintillation counting or passing through a Teflon coil for one of the continuous-flow methods. Photomultiplier pulse outputs are examined for coincidence and also summed. Should there be a coincidence, the summed pulse is subjected to pulse height analysis and those pulses falling within a preselected amplitude range are counted and recorded.

The differences between the two techniques which make for differences in the quality of the reported results derive from two factors. In the continuous-flow monitor the sample activity changes as a peak enters the measurement cell, proceeds through it, and finally exits. In addition, counting time tends to be short and for any given cell volume is fixed by flow rates. With the liquid scintillation counter, the sample is constant throughout the measurement and counting times are more flexible and can be established with due recognition to statistics. Just how do these differences affect the results?

One subject that we will barely touch upon is the question of counting efficiency. While some of that is a function of photomultiplier manufacture, a much bigger factor is the nature of the counting mixture and sometimes though less often, the sample itself. That the sample is frequently not involved ought not be surprising; in HPLC, sample is typically in pico/nanomolar concentrations. No matter; statistical expressions do not involve counting efficiency, but only observed counts and measured backgrounds and we will direct our attention to these.

Restricting our discussion solely to performance, the subjects we consider are:

1) Lower Limit of Detectability
2) Reproducibility

3) Accuracy and Confidence

4) Resolution

DISCUSSION

Lower Limit of Detectability

What is the least amount of activity that we can hope to see in a well-defined peak? A useful expression to help us make this estimate is:

$$LLD = 4.66 \times (Bkg/T)^{1/2}$$

where: *LLD* = Lower Level of Detectability, cpm

Bkg = background, cpm

T = measurement time, min

It tells us, with 95% confidence, that an observed count rate of *LLD* cpm is significant, i.e., that there is only a 5% chance of there not being true activity. Table 1 shows such values vs. different backgrounds and counting times; the backgrounds are considered representative of modern instrumentation and the counting times are believed to fall in the realm of practicality. Here, we also presume that in the samples subjected to the liquid scintillation counter, all the activity of a peak is contained in a single counting vial; as we shall later discuss under the heading of Resolution, that is a questionable proposition.

For any level of activity, a liquid scintillation counter with its static samples might be used to examine them for any time cited in our table, or even longer, the ultimate limit being set by throughput requirements or by the investigator's patience. It is obvious that as counting time increases, *LLD* decreases but the decrease is non-linear and to reach the lowest limits, very long counting times are required.

While the same statistics apply to the continuous-flow monitor, the same flexibility does not. Counting times are limited by cell volumes and flow rates. The reality is that large counting cells are rarely practical, 2.5 mL being the largest routinely shipped in commerce. Then, with eluate flow of 1 mL/min being the almost universal norm, and with a scintillator:eluate ratio of 3:1 at the lower limit of

TABLE 1

LLD vs. Counting Time and Background

	Counting Time – min					
	.25	.50	.75	1.0	5.0	10
Bkg-cpm	LLD					
10	29.4	20.8	17.0	14.7	6.6	4.7
20	41.7	29.4	24.1	20.8	9.3	6.6
30	51.0	36.1	29.5	25.5	11.4	8.1
	← Continuous-Flow			Liquid Scint. →		

practicality, residence time of .6 min (2.5/(3+1)) is about as long as can usefully be achieved. For counting with solid scintillators, essentially the same numbers hold true, i.e., the largest available packed cells have volumes of about 600 μL and with 1 mL/min of eluate flow, residence times again approximate .6 min.

We can now think about our table. In recognition of realities, it is divided in two parts, though, of course, there might be overlap. But, one who is a partisan of the liquid scintillation counter would not likely accept the short counting times forced by continuous measurement when there is advantage in employing longer counting times.

The conclusions are inescapable and anticipated: Lower background favors lower level counting. The liquid scintillation counter with its potential for longer counting times offers the possibility of measuring the lowest activity. And, implicit in these numbers but thusfar not discussed, is that higher counting efficiencies which thereby lead to more counts, also further low-level detection.

But, how much? When both can't be had, is it better to seek out more counts or lower background? Again, we should be able to approach the answer intuitively; statistical expressions involve counts^2/background and so it would seem that gaining sample counts is more beneficial than an additional background count is harmful. That premise is tested for representative continuous-flow and liquid scintillation counting systems:

TABLE 2

LLD -- Allowable Background vs. Count Rate Increase

0.5 Min Counting Time			5.0 Min Counting Time		
LLD	Bkg	Increase	LLD	Bkg	Increase
20.8	10.0	--	6.6	10.0	--
+10%	12.0	+20%	+10%	12.3	+23%
+20%	14.3	+43%	+20%	14.4	+44%
+40%	19.5	+95%	+40%	19.7	+97%
← Continuous-Flow			Liquid Scint. →		

If we begin with an LLD of 20.8 cpm for conditions of 10 cpm background and 0.5 min counting time and then determine what the allowable background would be for the 10, 20, and 40% count rate increases that might be attainable, we find 20, 43, and 95% increases with similar results for longer counting times with lower LLDs. Obviously, if one is looking for small peaks, it is better to work for a few more counts than it is to chase lower backgrounds.

Perhaps an even more constructive way to look at this picture is to recognize that the user has more latitude to gain counts than to reduce background. Especially in a continuous-flow monitor, the "background" is very largely photomultiplier crosstalk, accentuated by the proximity of one phototube to the other; the user has little control over the situation. But, the user can think about different counting mixtures, different scintillator ¦ eluate ratios, a different solid scintillator, any of which might add those few extra counts which can markedly improve the measurement.

Reproducibility

What might be expected in an attempt to reproduce results? The same relatively high activity sample (200,000 cpm.) was counted four times in a liquid

scintillation counter and gave results which reproduced to ± 1%; measuring the same material in a continuous-flow monitor after chromatography gave 3-minute wide peak integrals which reproduced only to ± 3%. Was anything wrong with the monitor, or is this to be expected?

We assume that monitor was operating properly as a counter; it was checked with sealed ^{3}H and ^{14}C sources which were counted repetitively. Different time segments were examined and a chi-square test was applied. The instrument counted within normal statistics.

Counting a fixed sample over and over in a continuous-flow monitor is not unlike counting a standard in a liquid scintillation counter. The entire sample is always under examination, not just a part of it, and small time segments are added up to make the complete counting time. Every time segment is subject to the same statistics and the error of the end result -- the square root of the sum of the squares of the errors of each individual measurement divided by the number of measurements -- is reduced as there are more time segments, i.e., a longer counting time.

But, the same is not true for a peak moving through the cell of a flow-through monitor. Taking our example of a 3-minute wide peak containing 200,000 cpm and breaking it down into 180 one-second segments, and further assuming, in order to simplify our analysis, that it is in the shape of an isosceles triangle, the center segment would contain 2,222 cpm while those at either end have near nothing. If the counting time -- the time of passage through the cell -- is 15 seconds, the center segment will give on average $2,222 \times {}^{15}/_{60} = 555$ cpm; 2 sigma is about 47 or 8.5%. But, if we look at a segment near either end, perhaps one that contains just 200 cpm, we measure only 50 counts in 15 seconds and 2 sigma is 14 or 28%.

Compare this situation with what would have been had we poured the two segments together, divided the resultant mixture in two, and then counted each segment separately under our same conditions. There would be 1,211 cpm in each segment ($2,222 + 200 = 2,422 \div 2 = 1,211$) and with each counted for 15 seconds, each would give on average 303 counts; 2 sigma is 35 or 11.5%.

Now, we can calculate a measure of statistical error. Applying the square root of the sum of the squares over n, in the first instance (8.5% and 28%) the combined error is 14.6% while in the second case (11.5% and 11.5%), the result is 8.1%. And, it will always be true that the minimum number comes about when all samples (in this

case the segments) are the same; when they are not, the result is always something greater. So, we have two problems with the flow-through monitor -- a fixed short counting time and a sample whose activity changes from one second to the next.

How does total activity level affect the situation? Suppose we had been working with one-tenth as much activity, i.e., 20,000 cpm rather than 200,000. Presumably, peak width which is largely a function of the chromatography would have been the same so each second of counting would show, on average, a 90% count rate decrease. In our flow situation, with one-second segments of 222 cpm and 20 cpm, the combined error computes to 46.7%. In contrast, had the two segments been combined and halved, each would have contained (222 + 20) ÷ 2 = 141 cpm and the combined 2 sigma error of counting both for 15 seconds each comes to 23.8%. At lower activity the errors are greater hence reproducibility is poorer.

While we have taken selected data points to accentuate the comparison, the tendencies cited are genuine. Had all 180 points of a three-minute wide peak been considered, the overall error in each case would have come to considerably less. But, the trends are correctly shown; all things being equal, the continuous-flow measurement is not as reproducible as is the liquid scintillation counter. Unfortunately, this is inherent in this form of measurement; it applies to all flow-through monitors and no manufacturer is immune. And, there is nothing that can be done about it. It will always be true that the liquid scintillation counter will give more reproducible results than the flow-through monitor, even if comparable counting times are employed.

Accuracy and Confidence

How good are the results we report? How accurate? How confident are we of the numbers we cite? In ordinary terms we might state that were we to inject n dpm into our column and obtain a result x, it is accurate to ± y % in z % of our experiments. If we define y and z, can x and n be calculated? And, if so, are there significant differences between the continuous-flow monitor and the liquid scintillation counter?

In the continuous-flow system, once a measurement cell with any given volume has been selected, the measurement time T secs is fixed by the combined flow rates of eluate and liquid scintillator (if used):

T = Cell Volume/Flow Rate

If the counting efficiency is E, the number of counts C actually obtained from n dpm is given by:

$$C = (T/W) \times n \times E$$

We begin with a simplification: for the fraction collector/liquid scintillation counter, the peak is W seconds wide containing n dpm and is collected and counted in a single vial. The average background is Bkg cpm. With the liquid scintillation counter, long background measurements are possible and the average background value is readily established. For continuous-flow, background runs may be made, or background may be measured between peaks during actual sample runs and a reasonable average value for current background ascertained; some systems permit this immediate average to be subtracted as a first step in any post-run calculation. It should be noted that constancy of background tends to be a greater problem with continuous-flow systems since they are more prone to contamination. In any case, the background B that might be accumulated while our peak of width W is being measured is:

$$B = Bkg \text{ cpm} \times (T \div 60) \times (W \div 60)$$

To establish sensitivity, we will solve for N, the number of counts required to give the desired performance level, using the expression:

$$T = (K/a^2) \times (1/N + 2B/N^2)$$

where:

a	=	required accuracy
K	=	4 (95% confidence level)
		1 (68% confidence level)
B	=	background per unit time
N	=	net counts per unit time

If we make some reasonable operating assumptions:

Peak width	=	60 seconds (W)
Bkg	=	10/20 cpm (Bkg)

TABLE 3

Performance Expectations

	Bkg = 10 cpm			Bkg = 20 cpm		
	LSC		Flow	LSC		
Conf. – %	Acc. – %	N	Acc. – %	Acc. – %	N	% inc.
95	± 5	1620	± 5.8	± 5	1639	1.2
68	± 5	419	± 5.9	± 5	437	4.3
95	± 10	419	± 11.6	± 10	437	4.3
68	± 10	117	± 11.9	± 10	131	12.0
95	± 20	117	± 23.8	± 20	131	12.0
68	± 20	38	± 25.5	± 20	47	23.7
95	± 50	28	± 64.6	± 50	35	25.0
68	± 50	11	± 69.9	± 50	15	36.4

Msmt. time – LSC = 60 seconds (*T*)

Msmt. time – C–F = 15 seconds (*T*)

we can construct a table comparing performance expectations for the continuous–flow monitor vs. the liquid scintillation counter.

The above table is interpreted as follows: Using the first row as an example, to achieve a 95% confidence level with an accuracy of ± 5% with a liquid scintillation counter having a background of 10 cpm, a sample in a counting vial must exhibit a count rate of 1,620 cpm. With that same activity level issuing from an HPLC column in the form of an isosceles triangular shaped peak just one minute wide passing through a continuous–flow monitor, and still assuming a background of 10 cpm, the accuracy of the measurement would drop to ± 5.8%.

Further, if the background of another liquid scintillation counter was 20 cpm, to achieve the same 95% confidence with ± 5% accuracy, would require a count rate

increase to 1,639 cpm or 1.2% more than with the lower background. No accuracy figures are given for a flow-through monitor at this second higher count rate and with higher background; they do not differ significantly from those calculated for the lower count rate and the lower background.

This information confirms what we intuitively know: At relatively high count rates, and with low backgrounds, the continuous-flow monitor can give results approaching those obtained with the liquid scintillation counter. But, as the count rate drops the performance of the continuous-flow monitor considerably diverges from that obtainable with the liquid scintillation counter; measurements are possible but quantitation suffers.

Resolution

How effective are the two methods in terms of preserving and reporting the separation that can achieved in the chromatography column? Here, there is not much comparison, the continuous-flow monitor being clearly superior. But, how much so? Is it possible to quantitate the quality of the separations?

In these discussions we must be careful not to compare apples with oranges. Fraction collection involves eluates as they leave an HPLC column; a typical peak might be 20-30 seconds wide. But, if that same peak were passed through a continuous-flow monitor under conditions which gave rise to a 30-second counting time, the measurement would show its width to be 50-60 seconds, the sum of true peak width plus the time for passage through the detector. Thus, when talking of peak width in one system it is not the same as in the other and we must allow for that.

With fractions being collected and counted in a liquid scintillation counter, the index time of the fraction collector must be less than the spacing between the two closest peaks, in fact, less than the time between the end of the first and the start of the next. If that can't be done with certainty, then any tube with activity might contain parts of two or more peaks. And, even if indexing is sufficiently frequent to preclude two peaks falling into the same tube, two adjacent tubes each with a separate peak can create the appearance of a single wider peak or, depending upon peak width, a single peak may be distributed into two or more tubes requiring that the ultimate counting results be summed.

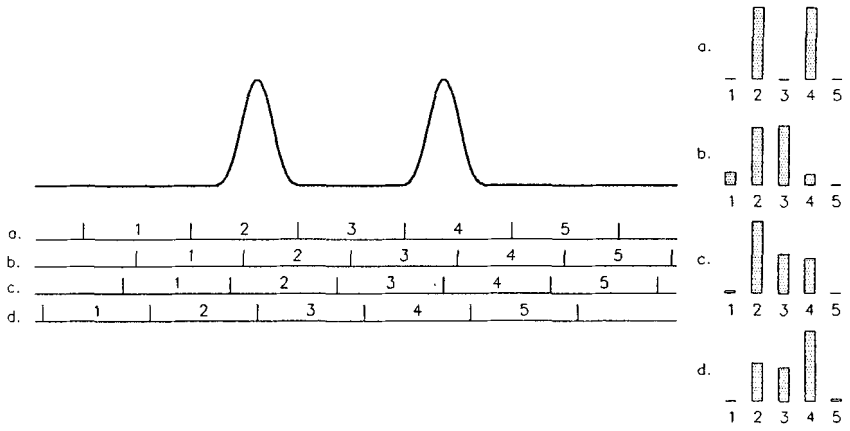

FIGURE 1. Fraction Collection of Closely Spaced Peaks.

The argument is made with the aid of a simplified illustration (FIGURE 1). Two relatively close peaks are shown together with four possible scenarios for fraction collection. In *a.*, the collection time falls exactly between the peaks and, as the peaks are narrower than the collection time, the five tubes illustrated perfectly define the two peaks. In the absolute worst case, *b.*, tubes 1 and 2 bisect the first peak and 3 and 4 almost bisect the second; the two peaks appear to be a single broad one. But, both of these situations are most unlikely. Much more probable is some variation of *c.*, or *d.*, where the randomness of fraction collection is evident and two peaks sometimes look like one and sometimes like two, but poorly resolved.

This is especially disadvantageous when the counts in a peak are low for even though the liquid scintillation counter is demonstrably more sensitive, with a peak divided into more than one counting vial, the effective background (over all the fractions) is multiplied while the counts are divided. Our discussion of LLD should be a reminder that a potential 50% (or more) count loss with constant background significantly lowers the expectation of being able to detect a small peak.

We might first consider the situation when a peak with our LLD of 20.8 cpm for a continuous-flow monitor with a background of 10 cpm, and with 30 seconds counting time is alternatively divided in half by the randomness of a fraction collector

and then counted in two vials. With each vial containing 10.4 cpm of activity, and with the liquid scintillation counter having a 10 cpm background and counting each vial for 5 minutes, the liquid scintillation counter shows to advantage. But, this is not reality. Precisely because peak division is apt to be random, there is a greater probability that one of the vials contains some amount of activity below the 5-minute LLD which therefore won't be seen, leading to an indeterminable error.

And, these comments assume good chromatography with perfect peak separation. The situation is even more uncertain with peaks that are not completely separated. Then, there will always be parts of two or more peaks counting as one. Shoulders will almost never be seen, nor will the fact of two peaks become evident unless tubes to either side of those containing the mixture count appreciably higher than do the tubes in the center.

What advantages does continuous-flow measurement offer? Peak spacing is less critical; if necessary, a small cell may be used though admittedly at the expense of LLD. Alternatively, if liquid scintillator is being used, the scintillator:eluate ratio may be increased to push the first peak from the cell before the second enters, again at the expense of LLD. While with fraction collection, smaller fractions might be taken to achieve similar results, as sample size is diminished overall problems increase. At the lowest levels of activity, there is greater likelihood of dividing a peak into parts, one or more of which fall below the LLD, overall consumption of counting fluid increases, and much more time is required -- including time for the measurement of regions of no activity between peaks -- to complete an experiment.

SUMMARY

Though statistical considerations -- essentially based upon the potential for an increased number of observed counts derived from longer counting times -- suggest that liquid scintillation counting offers the possibility of most accurately measuring low levels of radioactivity in HPLC eluates, a realistic appraisal does not wholly support that position. It has been shown that fraction collection/liquid scintillation counting likely produces the best counting results for well-defined, predictable, low-level peaks. On the other hand, when peaks are broad and precise location in time is not known, the uncertainties of fraction collection make that method unsuitable and the continuous-flow method clearly advantageous.

The reality, however, is that continuous-flow measurement has become the method of choice for radio-HPLC measurement for more subjective reasons -- time saving, reduced use of scintillator, simplified waste disposal, more convenient data processing -- and that its advantages (and disadvantages) in terms of the actual measurement are not significant factors in the overall picture.