

## Chapter 10

# A New Method of Sample Handling and its Application to Liquid Scintillation Counting Analysis

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The development of coincidence counters capable of reliable measurement of the weak  $\beta$ -emitting radioisotopes by liquid scintillation counting techniques made the use of radioactive tracers safe and simple. The first liquid scintillation counters were nothing more than replicas of one-channel  $\gamma$ -counters existing at that time. However, since their introduction 30 years ago, scintillation counters have benefited from many refinements in design which include sophisticated programmable features and computerised data processing.

In contrast, the original method of sample handling employing the counting vials was left unchanged and, as such, it remains the weakest link in the otherwise excellent analytical system. In fact, the use of counting vials has far more undesirable weak features than strong positive ones: (i) the fragility of glass counting vials continues to be responsible for the loss of samples and the main cause of costly decontaminations of laboratory tables, floors and instruments; (ii) glass (and plastic) vials have irregularities which usually cause optical distortions resulting in significant counting errors; (iii) low background counting vials must be made of high-quality, low in  $^{40}\text{K}$  content glass — consequently, such vials are expensive items; (iv) in order to maintain the highest possible counting efficiency and the lowest error, counting vials must be filled with large volumes of costly scintillation fluids; (v) the processing of samples in the counting vials requires a great deal of labour. After deposition of the sample in the vials, the vials must be filled with scintillation fluid, closed with screw caps, wiped clean and counted. Next, the vials must be removed from the counter, opened and emptied. Used vials, caps and the liquid matter must be collected in separate containers and carried to specific burial grounds. With the rise of material and labour costs, petroleum-product shortage and ecological pressures, the counting vial is a luxury which for most researchers is difficult to support.

These disadvantages provided the impetus which prompted a search for new ways to present the sample to the liquid scintillation counter. Many years of experimentation have led to an efficient system of sample handling which eliminates the use of counting vials, generates no solid wastes, considerably decreases liquid wastes and reduces the cost of analysis by a factor of ten.

It is well established that various parameters of compounds separated by chromatographic columns can be measured quantitatively employing different flow analysers. Most of the modern high-pressure, high-speed analysers are equipped with highly sensitive detectors and while using very small volumes of fluids, they can analyse submicrogram quantities of chemical compounds at a greatly accelerated rate.<sup>1,2</sup> Similarly, autoanalysers used in clinical laboratories can perform many analyses on samples of biological fluids transported in a discrete manner between gas bubbles without involving human hands.

However, the quantitation of radioactive compounds in the effluent of high-speed chromatographic analysers, or in the series of discrete samples supplied by the autoanalyser or other similar instruments, is a more difficult problem. In most such systems, submicrogram quantities of compounds are rapidly eluted in very small volumes. This essentially precludes collecting fractions for conventional analysis in counting vials or analysis in flow cells packed with solid scintillators.<sup>3-8</sup> One possibility is to mix the effluent with scintillation fluid and monitor the activity of the mixture in a scintillation counter by means of a hollow-tube flow cell.<sup>9-15</sup> This method of analysis is notably more efficient than counting in the flow cell filled with solid scintillators and therefore it is especially suitable for measurement of compounds labelled with tritium. However, because of drag along the wall at high velocity, the fluids flow significantly faster in the centre of small-bore conduits than along the wall (Fig. 1). This causes spreading of the separated components. When followed closely by another sample, the part of the sample flowing along the wall is overtaken by the centrally flowing part of the subsequent sample resulting in remixing of the samples. Introduction of air bubbles into the stream of liquid unifies the flow and prevents mixing of samples. However, air bubbles do not wipe off all of the passing liquid from the wall of the conduit and therefore they only reduce but do not eliminate the sample carryover.

It was found that if small segments of a semi-solid resilient gel are introduced into the conduit at constant intervals, the gel acts as a series of pistons that, for all practical purposes, maintain uniform flow along the wall and in the centre of the conduit. Furthermore, semi-solid spacer wipes off the traces of passing sample from the wall of the conduit far more efficiently than air bubbles. Because of this distinct action, such gel spacers as polyacrylamide or agarose can maintain discrete

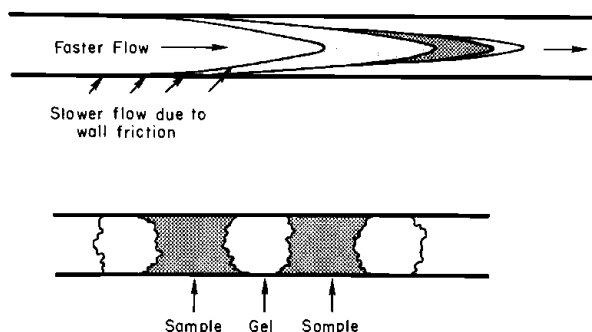


Fig. 1. Schematic illustration of wall effect and its elimination by the gel spacer.

transport of samples in long stretches of conduits. Also, because of the resilient nature, gel spacer glides freely through the restrictions, sharp turns and orifices in the apparatus and the interconnections without blocking them.

The basic apparatus used for the analysis of radioactivity in the effluent from a high-pressure amino acid analyser and of discrete samples is illustrated schematically in Fig. 2. Briefly, all interconnections were made using Cheminert tubing and connectors. The photometer of the amino acid analyser was connected with a piece of tubing to one port of a mixer. Another port of the mixer held a septum for manual injection of samples. The septum was connected to the spacer-gel pump, and to the piston 'a' of a Duplex Mini Pump. The third port of the mixer was connected to the piston 'b' of the Duplex Mini Pump. Both pistons of the Mini Pump were pumping multi-purpose scintillation fluid. The fourth port of the mixer was connected to the flow cell. The flow cell was mounted in a one- or two-channel liquid scintillation spectrometer which was equipped with a chart recorder and a printer. The outlet of the flow cell was connected to the waste reservoir. The details of this apparatus, materials and procedures are described elsewhere.<sup>14</sup>

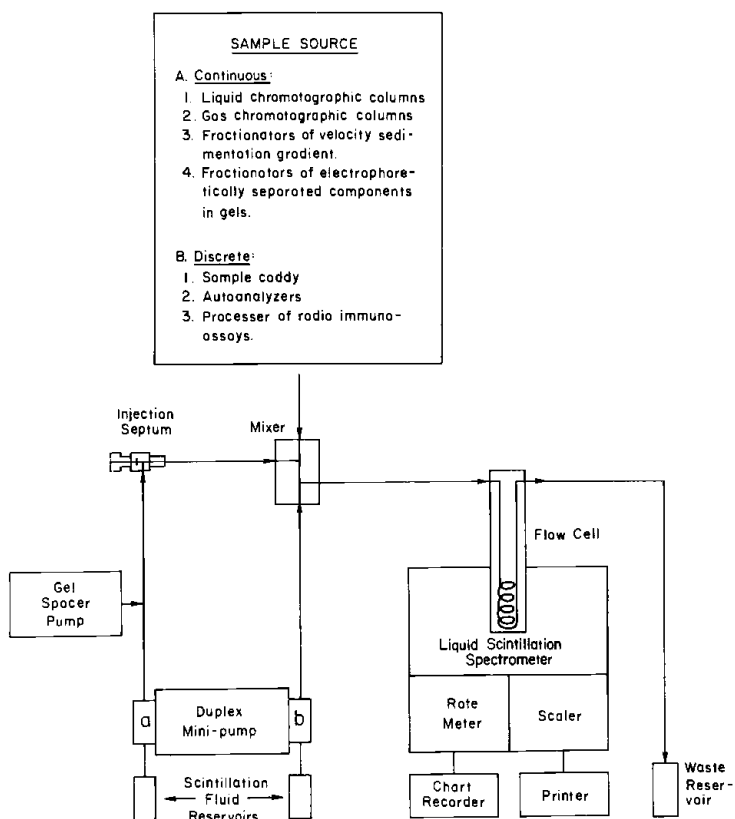


Fig. 2. Apparatus and connections used in analysis of discrete samples of effluent from amino acid analysis, and other sources.

The same apparatus was used for the analysis of individual samples, except that individual samples were introduced into the stream of flowing scintillation fluid and gel via a septum using a microsyringe, or samples were deposited with a microsyringe in a re-usable sample holder (Fig. 2). The sample holder was placed on a pneumatically operated sample caddy which, on command from a timer, moved the sample holder to the sample pick-up station. Once the sample holder was in place, the pumps were turned on and the sample was moved by the stream of scintillation fluid and gel spacer from the holder into the mixer. In the mixer, it was mixed with an equal volume of fresh scintillation fluid then directed into the flow cell and to the waste reservoir. Once in the flow cell, the sample was counted dynamically without stopping the pumps, or the pumps were stopped and the sample was counted for either a preset time interval or a preset error. Also, in the stop and count mode, the efficiency of counting was determined automatically with an external standard. The mode of counting can utilise essentially all features of all modern counters.

The effects of the spacer gel on the measurement of the radioactivity in the flowing stream of liquid are illustrated in Fig. 3. Twenty, 15, 10 and 5  $\mu\text{l}$  aliquots of a solution containing 741.5 disintegrations  $\text{min}^{-1}$  for each microlitre of  $^{14}\text{C}$ -leucine were injected at 45 s intervals with a microsyringe through the septum into the stream of scintillation fluid and analysed in a 1.06 ml flow cell at a rate of 3.45  $\text{ml min}^{-1}$ , without adding polyacrylamide spacer gel. The same experiment was performed in the presence of gel spacers.

When the spacer gel was left out, each sample needed 48 s to clear the flow cell. Also, it generated a peak with a long trail. As a result, samples injected in succession produced a profile of fused peaks. This indicated that the front of the oncoming sample caught up with the preceding sample and caused mixing of the samples.

However, when the same samples were analysed in the presence of the spacer gel, the sample cleared the flow cell without trailing. This is seen especially clearly in the profile produced by samples injected in rapid succession. Each sample formed a sharp peak separated by a valley dropping down to the baseline.

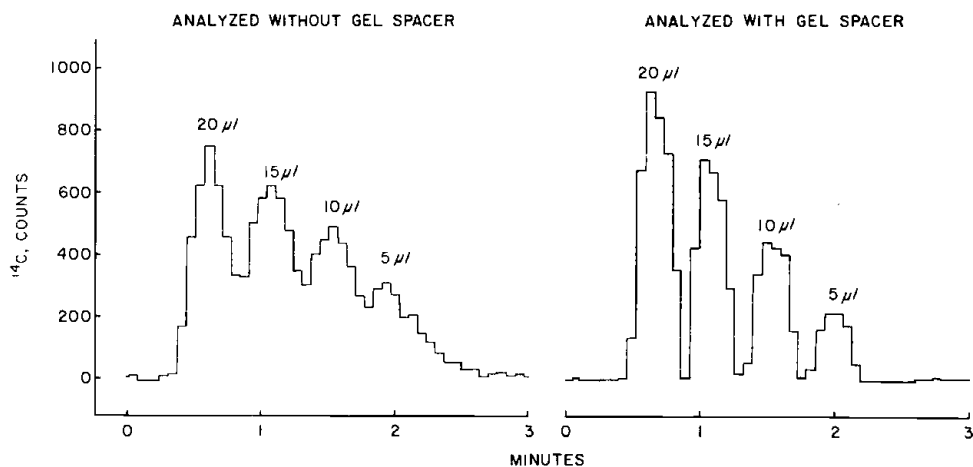


Fig. 3. Effect of spacer gel on the transport of samples in conduits of small diameter.

Measurement of radioactivity in the samples delivered by the sample caddy produced similar results (Fig. 4). Series of 5, 10, 20, 25, 30, etc.  $\mu\text{l}$  aliquots of  $^{14}\text{C}$ -leucine standard, containing  $741.5$  disintegrations  $\text{min}^{-1}$  for each microlitre, were deposited with a microsyringe into the sample holder and placed on the sample caddy. The sample caddy was programmed to change the samples at 2 min intervals. The analysis was done in a 1.06 ml flow cell at  $3.2$  ml  $\text{min}^{-1}$  liquid flow. The activity was documented by the chart recorder and by the printer.

As shown in the top portion of Fig. 4, the size of the peaks were proportional to the size of the analysed aliquots. Also, each sample generated a sharp peak which dropped to the natural background without trailing. As shown on the bottom of Fig. 4, the plot of numerical values versus corresponding aliquots of standard produced a straight line. Data accumulated by repeated analysis of the same samples

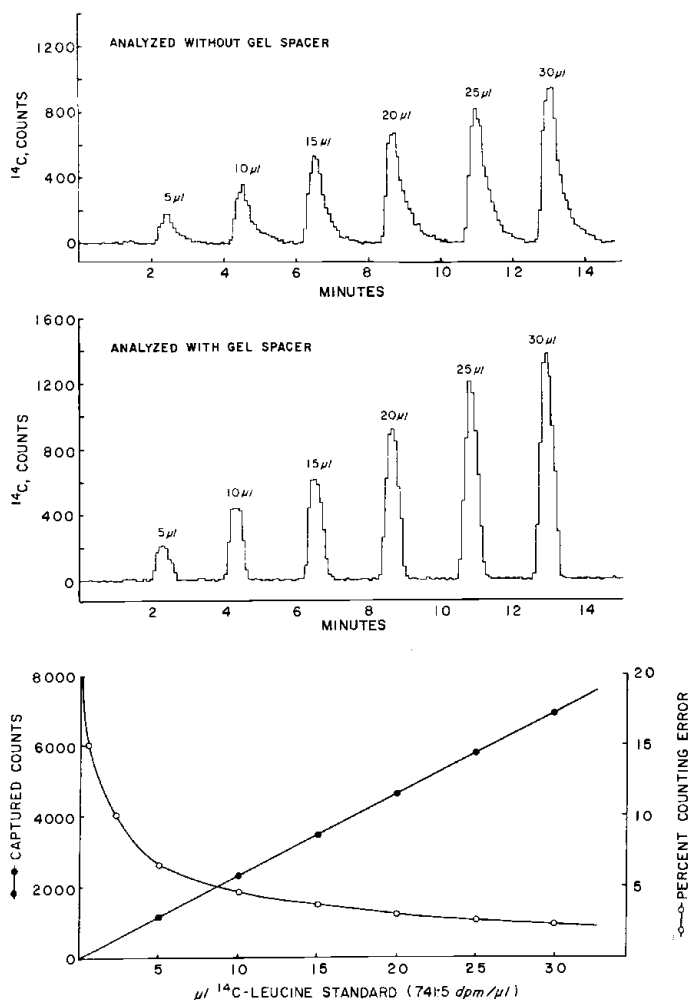


Fig. 4. Effect of spacer gel on the analysis of discrete samples delivered by an automatic sample caddy.

revealed that the counting error for the aliquot containing 500 disintegrations  $\text{min}^{-1}$  was  $\pm 11\%$ , for 1000 disintegrations  $\text{min}^{-1}$  was  $\pm 7.3\%$ , and that for 20,000 disintegrations  $\text{min}^{-1}$  was  $\pm 1.6\%$ . These figures are in complete agreement with values obtained in counting vials. Of course, greater counting error of less active samples is not due to the lack of precision of sampling or loss of the sample, but to uneven decay of the isotope.

The efficiency of counting of isotopes in the flow cell was calculated by using the equation which takes into account the rate of fluid flow and volume of the flow cell:<sup>15</sup>

$$E (\%) = \frac{(100 \%) \times (\text{rate of flow, ml min}^{-1})}{(\text{disintegrations min}^{-1} \text{ per sample}) \times (\text{millilitres per flow cell})}$$

$$\times (\text{count per peak}) - (\text{background per peak})$$

When the data obtained for the standards were substituted in this equation, the efficiency of counting of  $^3\text{H}$ -leucine and  $^{14}\text{C}$ -leucine in a 1.06 ml flow cell in the above described experiment was 51% and 94.3%, respectively.

This equation shows that in continuous flow analysis, the rate of flow and volume of flow cell are critical parameters and require careful consideration for achieving optimal counting conditions.<sup>15</sup>

The results of measurements of radioactivity in the effluent of a high-pressure Durrum D-500 Amino Acid Analyser are shown in Fig. 5. In this experiment, the amino acid analyser was programmed to analyse protein hydrolysate. The column was charged with 20  $\mu\text{l}$  of the amino acid calibration mixture containing 8 nmol (each) of 16 non-radioactive, and 7 to 70 pmol of 15  $^{14}\text{C}$ -labelled amino acids. The amount of radioactivity, contributed by individual amino acids, varied from 4800 to 7200 disintegrations  $\text{min}^{-1}$ . The analysis was carried out in a 0.53 ml flow cell at a total flow rate of 4.23  $\text{ml min}^{-1}$ . The analysis was completed in about 55 min. As shown in Fig. 5, tracings of ninhydrin colour, recorded by the photometer of the D-500 Analyser, and of radioactivity, recorded by the scintillation spectrometer, showed essentially the same degree of resolution. They differed only in respect to the number and height of peaks. As expected in the tracings produced by the photometer, the height of the peaks was proportional to the amount of each amino acid; in the tracings of the scintillation spectrometer, they were proportional to the amount of radioactivity present in each amino acid. Furthermore, the scintillation spectrometer detected presence of proline, while the D-500 Analyser did not because the photometer, operating at 560 nm, could not detect colour produced by ninhydrin reacting with proline. In contrast, the D-500 Analyser detected cystine, methionine and ammonia which were non-radioactive. The separation of amino acids emerging within 30 s of each other, e.g. serine-threonine of isoleucine-leucine, was the same in both tracings. The profiles differed mainly in that the photometer tracings consisted of a smooth line while that of the scintillation counter, recorded at 1 mV sensitivity, consisted of a characteristic jagged line reflecting random isotope decay. The efficiency of counting  $^{14}\text{C}$  in this experiment was about 68%. It should be noted that complete analysis consumed 220 ml of scintillation fluid. To the best of my knowledge, no other system can produce such results.

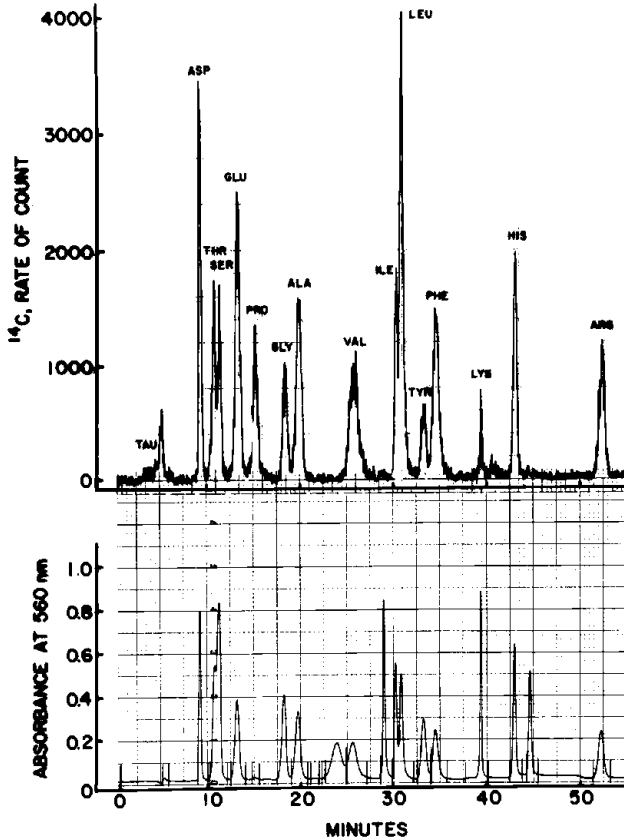


Fig. 5. Tracings of radioactivity and ninhydrin colour in the effluent from the Durrum D-500 Amino Acid Analyser.

In addition to analysis of individual samples or column effluents, this system is well suited for analysis of samples presented by a wide variety of analytical devices. Thus far, this system has been used to measure radioactivity of protein and nucleic acids separated by electrophoresis in gels,<sup>16</sup> and in velocity sedimentation gradients.<sup>17</sup>

Promising results were also obtained with gas chromatographic and nucleic acid analysers.<sup>18</sup> As the function of the gel spacer is similar to that of the air bubbles, the system can be adapted for clinical colorimetric assays – performed by Technicon's Autoanalyzer – and radio-immuno assays. In fact, gel spacer is not subject to compression, it does not lose its effectiveness with change of pressure or temperature, and therefore it may have a wider range of application than the air bubbles.

The composition of the spacer is especially important because it governs the effectiveness of separation of the samples and cleaning of the inner walls of the conduits. The spacer must be compatible with the liquid scintillation counting process; it must be inert enough not to absorb or interact with either the sample or the scintillators; and its physical properties should not change when it is mixed with the scintillation fluid or the sample. Of the number of semi-solid resilient materials tested, polyacrylamide gel fulfilled these requirements the closest. It is an effective

spacer in concentrations between 3 to 7 g per 100 ml. However, it is most effective in concentrations between 5 and 7 g per 100 ml. It is also most effective when introduced in small amounts at 2 to 3 s intervals. The amount of spacer gel needed to maintain discrete transport depends on the rate of flow and diameter of the conduit. In the experiments described above, 40 to 60  $\mu$ l of 7 g per 100 ml gel introduced in 31 segments per minute maintained discrete flow quite adequately.

The other critical factor is the composition of scintillation fluid. However, a number of commercially available multi-purpose scintillation fluids are suitable for flow analysis. Many of these products are formulated in such a way that they are equally suitable for the aqueous and non-aqueous samples.

As is evident from these experiments, this system of sample transport can satisfy the requirements of essentially all types of isotope measurement and thus it eliminates the need for counting vials. Furthermore, it can be adapted for various other analytical purposes.

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## REFERENCES

1. S. Moore, in *Chemistry and Biology of Peptides*, Proc. 3rd Amer. Peptide Symp. (ed. J. Meienhofer), Ann Arbor Science Publishers, Inc., Ann Arbor, Michigan, 1972, p. 629.
2. H. K. Knox, *Lab. Practice* **22**, 52 (1973).
3. E. Schram and R. Lombaert, *Anal. Chim. Acta* **17**, 417 (1957).
4. D. Steinberg, *Nature (London)* **182**, 740 (1958).
5. D. Steinberg, *Nature (London)* **183**, 1253 (1959).
6. B. L. Funt and A. Hetherington, *Science* **129**, 1429 (1959).
7. E. Schram and R. Lombaert, *Anal. Biochem.* **3**, 68 (1962).
8. K. Piez, *Anal. Biochem.* **4**, 444 (1962).
9. K. H. Clifford, J. W. Hewett and G. Popjak, *J. Chromatog.* **40**, 377 (1969).
10. H. W. Scharpenseel and K. H. Menke, in *Tritium in Physical and Biological Sciences*, Vol. 1, International Atomic Energy Agency, Vienna, Austria, 1962.
11. J. A. Hunt, *Anal. Biochem.* **23**, 289 (1968).
12. L. Schutte, *J. Chromatog.* **72**, 303 (1972).
13. D. R. Eyre, *Anal. Biochem.* **54**, 619 (1973).
14. B. Bakay, *Anal. Biochem.* **63**, 87 (1975).
15. B. Bakay, *Clin. Chem.* **21**, 1212 (1975).
16. B. Bakay, *Anal. Biochem.* **40**, 429 (1971).
17. B. Bakay, unpublished.
18. W. Weyler, L. Sweetman and B. Bakay, unpublished.

## DISCUSSION

**P. Johnson:** First I must congratulate you on your fascinating technique. Could I ask whether your method has any limitations with regard to the types of solvent or the pressures that may be used?

**B. Bakay:** We have used all types of aqueous systems and non-aqueous organic solvents with no problem. The full range of pressures normally used in high pressure liquid chromatography is acceptable.

**P. Tothill:** I should like to congratulate Dr. Bakay on an extremely elegant and novel development and ask whether the flow rate through the counter has to be compatible with the output of the analyser. Is it possible to have a longer counting time than the interval between the effluent samples?

**B. Bakay:** Yes. When the pumps are stopped there is no movement or mixing in the system.

**B. Fox:** I did not fully appreciate from your excellent paper whether the gel spacer itself contained scintillant or not.

**B. Bakay:** No it did not. We have considered it and can do it, but do not think that there would be any advantage.