

SOME FACTORS INFLUENCING  
EXTERNAL STANDARDIZATION

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ABSTRACT

A study has been made of some factors which effect the external standard ratio-efficiency curve for samples counted in a Searle Isocap 300 liquid scintillation spectrometer fitted with lesser pulse height analysis. The effect of changing the volume of a simple scintillation solution was investigated and it was shown that little difference occurs in the external standard ratio-efficiency curve when chemically and colour quenched tritium and chemically quenched  $^{14}\text{C}$ -samples were studied whereas substantial differences were apparent for colour quenched  $^{14}\text{C}$ -samples. In addition a study has shown that the percentage standard deviation of the external standard ratio for background samples under a range of conditions is less than 0.75% while for samples containing high activity ( $10^6$  DPM) the value rises as high as 2.8%.

INTRODUCTION

Although automatic external standardization was introduced more than a decade ago (1-2) there is still considerable reluctance among some users of liquid scintillation spectrometers to employ this useful technique to assess the counting efficiency of their samples and there is yet another group who use it in less than the

proper manner. Some of the reticence is no doubt well founded firstly because of certain shortcomings in the mechanisms of early units and a lack of understanding of the technique which led to experiences which are not readily dispelled even though modern units are very reliable. Secondly, some publications describe the use of the procedure for samples which are heterogeneous or slowly soluble or which adhere or are adsorbed to the glass wall of the vial. Under such conditions the method can give very unreliable results. In addition, the correct use of external standardization with scintillation solutions containing surfactants requires that the standard curve be generated using a quenching agent which is the same or very similar to that encountered in the sample. In addition the proportions of sample, surfactant and scintillation solution should be carefully controlled if reliable results are to be obtained. Thirdly, possibly as a result of commercial promotion, the procedure of external standardization is often considered to be "independent" of sample volume and quenching agent. Thus workers sometimes do not ascertain that this is indeed the case for their samples and the spectrometer they use. Fourthly, some workers use one calibration curve, derived perhaps from the set of quenched standards supplied with a new instrument, and apply it to all samples for many months (or years) without revalidating it or checking for instrument drift. The particular scintillation solution used in the standards and its resistance to quenching may be quite different from the one the worker is currently using. The results which are generated are thus likely to be incorrect. Fifthly, the nature of the processes which give rise to chemical and colour quenching are quite different (3-7) and the external standard ratio vs counting efficiency must be investigated for the instrument being used, the settings of the pulse height analyzers and the quenching agents. Sometimes two quite different curves arise especially with isotopes with  $\beta$ -energies similar to or greater than carbon-14.

Since there is a dearth of published information on this subject the present investigation was undertaken to look carefully at a few of these areas, namely, the effect of the volume of the sample on its counting efficiency and external standard ratio for both the chemical and colour quenched samples containing tritium or carbon-14. In addition a study was made of the reproducibility of the external standard ratio for samples having no activity as

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compared with those containing very high activity and assessment was made of the reproducibility of the external standard ratio value and its relationship to the geometry of the scintillation vial.

### MATERIALS

#### *Instrument*

The liquid scintillation spectrometer used was a Model 6872 Searle Analytic Isocap 300 with a temperature controlled counting cabinet operated at 20.5°C. The instrument was fitted with an external standard consisting of a barium-133 source positioned automatically beneath the sample vial using a rigid tungsten rod. Live timing and synchronous counting facilities were fitted to the spectrometer. The latter causes the timer to continue, after a preset count has been reached, until the next increment of the printed time has elapsed. This permits more accurate assessment of counting rates for high activity samples especially when they are measured for short preset times.

The liquid scintillation spectrometer was fitted with a photon monitor, cross-talk discriminators (8) and pulse analysis was carried out by the lesser pulse height procedure (9,10). The two analyzers were set as follows:-  
TRITIUM - CAPS 4 Ch. A = 0.5 → 9 and Ch. B = 0.5 → 18 keV, external standard ratio channels (ESR) Ch. A = 5.6 → INF and Ch. B = 0.5 → 18 keV.  
CARBON-14 - CAPS 5 Ch. A = 1.2 → 60 and Ch. B = 4.5 → 150 keV, ESR Ch. A = 0.5 → 18 keV and Ch. B = 3.6 → INF keV. The instrument was calibrated (CAPS L position) two days before this study commenced and the calibration was checked daily over the four weeks during which the work was performed. The instrument was used exclusively for this study and no change in calibration was required.

#### *Scintillation solution*

The scintillation solution contained 8.00 g PPO made up to one litre with toluene. Its density at 20°C was taken as 0.866 g/ml. PPO (M.Pt. = 71°C) was obtained from Ajax Chemicals, Australia, and toluene (Boiling Range 109.5 - 111.5°C) was Pronalys Grade and obtained from May and Baker, Australia. Both were used without further purification.

### *Quenching Agents*

Nitromethane, was obtained from Ajax Chemicals Australia and used as the chemical quenching agent. Its density was taken to be 1.13 g/ml.

The colour quenching agent was  $\beta$ -carotene, obtained from Koch-Light Ltd. U.K., prepared as a 20 mM solution in the standard scintillation solution. Its density was measured crudely to be 0.868 g/ml. Prior to use it was filtered through a Whatman No. 1 paper.  $\beta$ -carotene was chosen in preference to the more commonly used methyl orange since the latter gives solutions which are not optically stable.

### *Standards*

Tritiated hexadecane and  $^{14}\text{C}$ -hexadecane were obtained from The Radiochemical Centre, Amersham, U.K. and has activities with estimated overall uncertainties of  $\pm 3\%$  and  $\pm 2\%$  respectively. The density of hexadecane was taken as 0.773 g/ml and  $20^\circ\text{C}$ . For the short study involving high activity samples, large volumes (up to 1.5 ml) of hexadecane could not be used since considerable dilution of PPO would have occurred. Instead  $^3\text{H}$ -toluene and  $^{14}\text{C}$ -toluene standards ( $\pm 1\%$  and  $\pm 3\%$  respectively), obtained from Packard Instrument Inc., U.S.A., were used and PPO was added to obtain a concentration of 8 mg/ml (8 g/litre).

### *Scintillation vials*

Glass scintillation vials were obtained from Packard Instrument Inc. and these were fitted with foil lined lids. Sufficient vials for the study were selected as being similar on the basis of their outer dimensions and weight (see reference 5 for details). Light transmission within the batch had a standard deviation (S.D.) = 0.75% as judged by counting rates observed when they contained 20.0 ml of a standard scintillation solution spiked with tritium ( $10^5\text{DPM}$ ) and then counted to 800,000 counts. External standard ratios using the tritium CAPS setting had a S.D. = 0.9%. The vials were then washed before use in the present study.

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### METHODS

Two volumes of scintillation solution were labelled, one with tritiated hexadecane and the other with  $^{14}\text{C}$ -hexadecane. Standard hexadecane, placed in a small tared vessel, was weighed to within a 0.1 mg and then quickly transferred to 350 ml (checked by weighing) scintillation solution. Thus a solution containing an accurately known activity (about  $10^5$  DPM per 20 ml) was obtained. From each of these solutions ten aliquots of 30 ml were transferred to tared 50 ml glass tubes having close fitting stoppers. Speed was essential to avoid evaporation of the toluene. The tubes were then reweighed and any contents exceeding an error range of  $\pm 0.15$  ml were readjusted to come within this tolerance. Each set was used in the study of chemical quenching and after this had been completed (about two weeks) another ten aliquots of each standard scintillation solution were prepared and used in the investigation concerning colour quenching.

Nitromethane and  $\beta$ -carotene solution were added as predetermined volumes to the 30 ml aliquots of scintillation solution. These volumes were derived from a pilot study and for nitromethane up to 150  $\mu\text{l}$  for  $^3\text{H}$  and up to 390  $\mu\text{l}$  for  $^{14}\text{C}$  were required while for the  $\beta$ -carotene solution the respective volumes were 72  $\mu\text{l}$  and 155  $\mu\text{l}$ . Gilmont precision microlitre pipettes were used for dispensing the quenching agents. Since these pipettes were calibrated "to contain" they were rinsed twice in the radioactive solution and then cleaned before being used for the next sample.

A Kontes dispenser was used to transfer by glass pipette, 5 ml of the quenched standards to the selected and weighed ( $\pm 1$  mg) scintillation vials which were then capped and reweighed to obtain the weight of the scintillation solution and hence the DPM of the hexadecane contained therein. Appropriate allowance in this calculation was made for the volume of added quenching agent. The vials were then temperature equilibrated in the spectrometer with its lid closed so as to preclude problems of chemiluminescence or phosphorescence of the glass vials. Each sample was then counted at least twice for twenty minutes or 200,000 counts ( $1\sigma = 0.22\%$ ) together with the external standard cycle appropriate to the CAPS setting. The vials were then reweighed and generally less than 10 mg loss was observed and it was therefore assumed that since

hexadecane has a very low vapour pressure the loss was mainly due to toluene and perhaps a little nitromethane. Consequently, it was taken that there had been no loss of radioactivity or change in quenching. Using this new starting weight a further 5 ml aliquot of the same quenched standard was added so that the vial now contained 10 ml. The counting process was then repeated and the overall procedure reproduced with the same vials next containing 15 ml and then 20 ml of the quenched standard solutions.

#### *High activity samples*

A predetermined volume of scintillation solution was placed in a weighed vial and then sufficient (0.5 → 1.5 ml) of standard toluene (labelled either with  $^3\text{H}$  or  $^{14}\text{C}$  and containing 8 mg/ml PPO) was added to give around  $10^6$  DPM. Actual DPM was obtained by weighing. An air quenched sample was evaluated (see next section) as were samples which had been quenched with the nitromethane or  $\beta$ -carotene. The total volume of sample was 20.0 ml.

#### *Reproducibility of external standard ratio values*

The scintillation spectrometer was first modified so that when a sample was kept loaded it was possible to obtain repeated evaluations of the external standard ratio. A sealed unquenched standard background sample was used and was kept in the detector in a constant position while thirty external standard ratio evaluations were made using the CAPS 4 (tritium setting). Then with the same CAPS setting thirty external standard evaluations were performed with the vial being loaded on each of thirty cycles of the sample belt. Thus, in the first procedure the reproducibility of the  $^{133}\text{Ba}$  external standard source could be assessed since the geometry of the vial was kept constant while in the second the variation the vial geometry, together with the positioning of the external standard could be measured. This procedure was repeated on the same samples using the CAPS 5 setting for carbon-14.

A similar study was made for background samples which had been either heavily chemical or colour quenched.

The high activity samples, prepared as described above, were also subjected to a study in which the reproducibility

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of the external standard ratio was assessed while the vial was kept in the loaded position. Five observations were made on each sample.

### RESULTS AND DISCUSSION

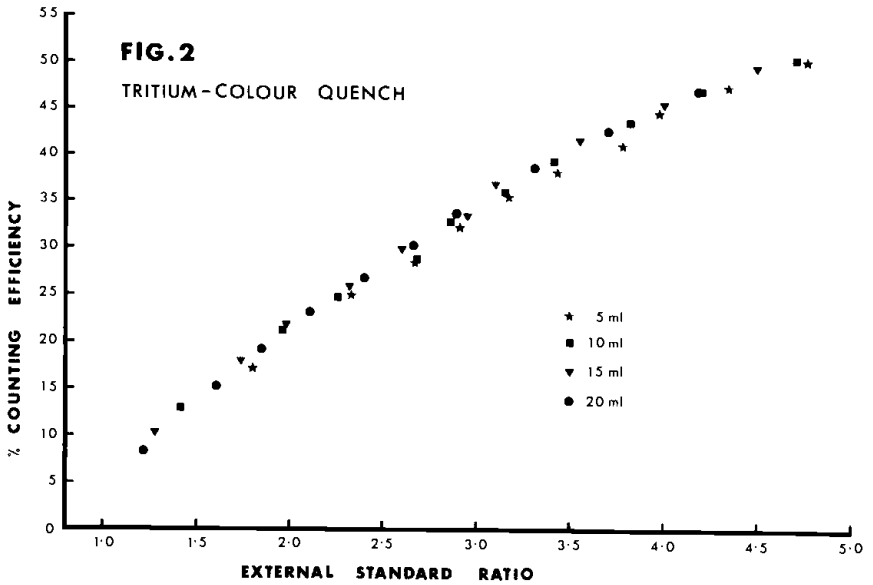
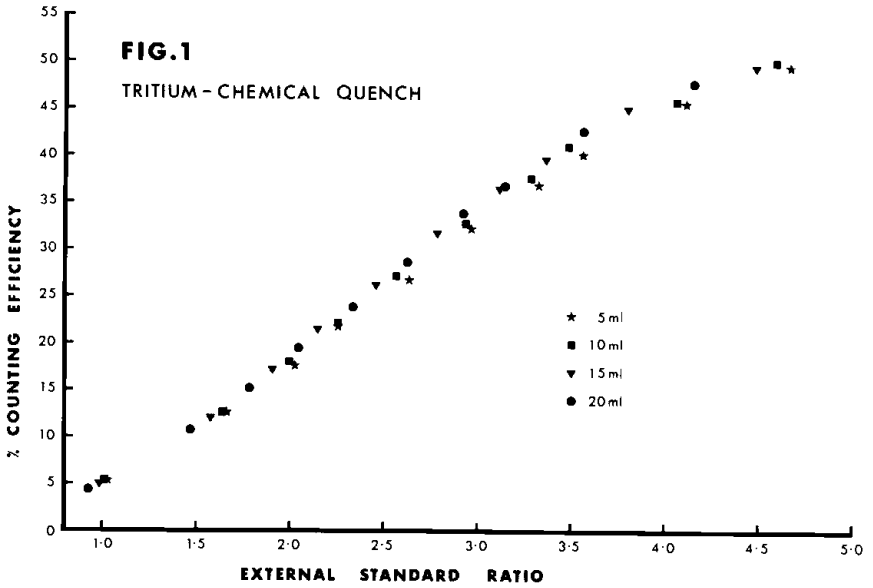
The effect on the ESR-efficiency curve when different volumes of sample were appraised is presented for tritium in Fig. 1 (chemical quench) and Fig. 2 (colour quench) and for carbon-14 in Fig. 3 (chemical quench) and Fig. 4 (colour quench).

In the spectrometer used for this study the external standard windows are very similar for tritium and carbon-14 (see *Instruments*) and the form of the ratio taken for tritium is simply the reciprocal of that for carbon-14. Thus the curves of the two radionuclides are related in an inverse manner.

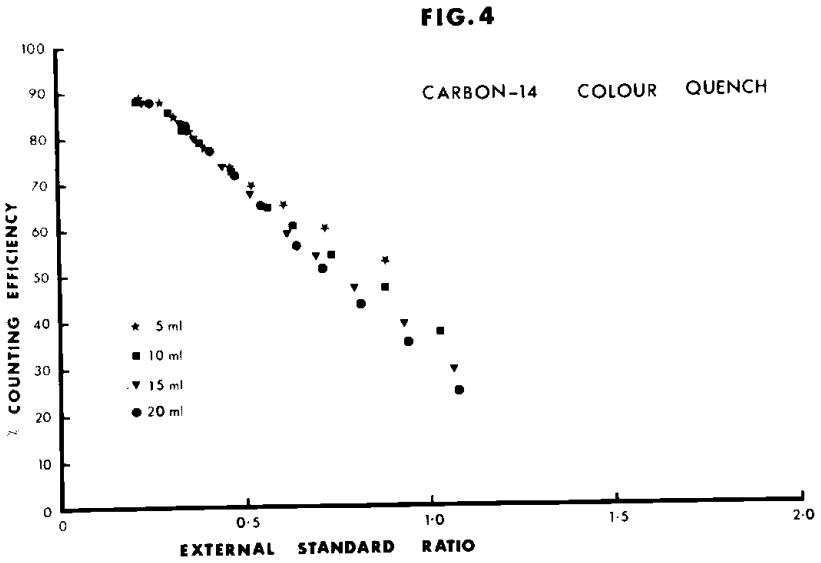
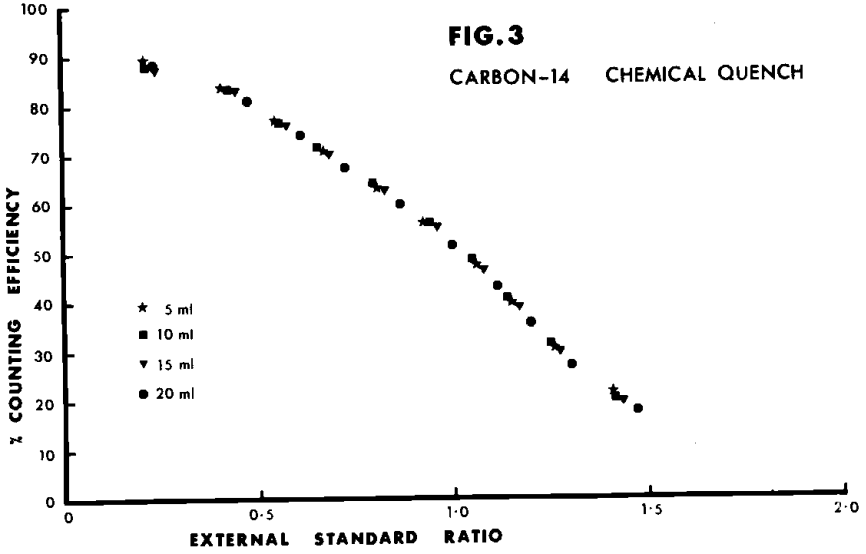
Consider first the results for tritium (Figs. 1 and 2). All the points lie more or less along the same line with a maximum spread of about  $\pm 1.5\%$  in the counting efficiency for both the chemical and colour quenched samples. The outlying points are mainly from the 5 ml sample size. Thus for most purposes the curve for the chemically quenched samples and for the colour quenched samples can be regarded as being reasonably independent of sample volume between 5 and 20 ml. The two curves themselves are very similar for sample efficiencies above 30% but below this value they diverge and samples containing both chemical and colour quenching agents would be difficult to assess with accuracy.

The results for the chemically quenched samples of carbon-14 (Fig. 3) show that the ESR-efficiency curves for the four different volumes can be completely super-imposed and thus they can be considered as being independent of sample volume between 5 and 20 ml. Like the tritium samples the ESR values and efficiencies do change for the various volumes of sample but the points simply move along the curve.

Colour quenched samples containing carbon-14 (Fig. 4) show a separate relationship between ESR and efficiency for each of the four volumes studied. The curves are similar for efficiencies above 70% but diverge significantly below



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this value. Note that the ESR value for the tenth (most quenched) sample of 5 ml volume lies between the eighth and ninth sample of 20 ml volume. This extreme value would be read as an efficiency of 39% on the curve for 20 ml samples whereas in reality it is 53%.

Why then do the tritium samples not behave in the same manner as the carbon-14? The explanation hinges on two facts. Firstly for samples of different volumes the photons must travel different pathlengths to escape from the vial. In colour quenching the photons are absorbed by the agent and the absorbance is related exponentially to the path length. Secondly the energy of the radionuclide has a considerable influence since for an average energy (50.2 keV) carbon-14  $\beta$ -event some 800 photons are produced whereas for tritium (average energy 5.6 keV) around 65 are generated. The results generated from a computer simulation of the liquid scintillation process (5) indicate that for unquenched carbon-14 the number of photoelectrons produced at the photocathode of the photomultiplier is around 60 whereas for tritium the number is about 4. Thus for tritium, from the statistical viewpoint, little variation is possible in the pulse height since at least two coincident photoelectrons are required to enable coincidence and to register a single count. However for carbon-14 with 60 photons per average event there is more statistical variation within the pulse heights due to the stochastic nature of the overall liquid scintillation process and more particularly in this case with the path lengths travelled by the photons and whether or not they are absorbed by the colour quenching agent. Due to the larger number of photoelectrons there is also of course a better chance of enabling coincidence and registering a count.

Thus all colour quenched, carbon-14 samples should have the same volume as the standards. Since the chemical quench ESR-efficiency curve does not lie close to any of the colour quench curves the determination of efficiencies would appear to be precluded for  $^{14}\text{C}$ -labelled samples containing both chemical and colour quenching agents.

The results of the study on the reproducibility of ESR on background samples is presented in Table I. It can be seen that the ratios obtained when the vials were kept in the loaded position are almost identical to those obtained when the vials were loaded once on each cycle of the sample

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TABLE I

## REPRODUCIBILITY OF ESR ON BACKGROUND SAMPLES

CAPS 4 (TRITIUM + ESR)

	<u>Vial Kept Loaded</u>		<u>Vial Cycled</u>	
	ESR	% S.D.	ESR	% S.D.
Unquenched	5.24	0.62	5.28	0.71
Heavy chemical quench	0.93	0.38	0.93	0.34
Heavy colour quench	1.28	0.42	1.28	0.48

CAPS 5 (CARBON-14 + ESR)

	<u>Vial Kept Loaded</u>		<u>Vial Cycled</u>	
	ESR	% S.D.	ESR	% S.D.
Unquenched	0.191	0.70	0.191	0.69
Heavy chemical quench	1.520	0.43	1.520	0.27
Heavy colour quench	1.020	0.26	1.020	0.31

ESR = External Standard Ratio  
n = 30

TABLE IIREPRODUCIBILITY OF ESR ON SAMPLES CONTAINING  $10^6$  DPMCAPS 4 (TRITIUM + ESR)

		% Efficiency	ESR	% S.D.
Air Quenched		52.0	4.42	2.84
Chemical Quenched	1	41.7	3.27	1.97
	2	32.8	2.69	0.95
	3	17.7	1.84	0.60
	4	5.0	0.94	0.50
Colour Quenched	1	34.1	2.75	1.79
	2	23.1	1.97	0.96
	3	11.0	1.30	0.68

CAPS 5 (CARBON-14 + ESR)

		% Efficiency	ESR	% S.D.
Air Quenched		88.0	0.227	1.46
Chemical Quenched	1	51.7	0.996	0.53
	2	17.4	1.490	0.52
Colour Quenched	1	58.5	0.608	0.84
	2	29.7	1.014	0.96

ESR = External Standard Ratio

n = 5

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belt. The standard deviations are small and similar in magnitude. Thus the geometry of the vials used and their positioning in the detector have little or no effect on the external standard ratio. In a previous study using a different model of spectrometer and different external standard the present author reached the conclusion that the vial and its position in the detector played a small but significant role in determining the ESR (11).

It is sometimes held that high sample activities cause perturbations in the ESR presumably due to pulse pile up in the analyzers. Table II shows the results of a study designed to investigate this problem. As can be seen larger standard deviations are recorded for high activity samples. Those samples which are quenched show a diminishing percentage standard deviation and this is consistent with the hypothesis that the pulse pile up is occurring. Air quenched samples containing  $10^5$  DPM had a S.D. = 0.64% for tritium and 0.82% for carbon-14. Further work would be necessary to ascertain the threshold counting rate at which the S.D. of the ESR starts to increase. However it is worth noting that for the maximum error observed the S.D. = 2.84% and this is not very significant in real terms since it represents only about one percent change in the counting efficiency and for most purposes this could be neglected.

## CONCLUSION

In many applications and especially the double label situation external standardization offers the only convenient and accurate means of assessing counting efficiency. To the author's knowledge there is no standard published procedure to which a beginner can refer to obtain the details necessary for drawing up an ESR-efficiency curve. While the method described herein is not put forward as a protocol it is hoped that it will act as a nidus for the establishment of standard protocols in liquid scintillation spectrometry.

The results of the investigation indicate that the reproducibility of the external standard ratio for the instrument investigated is very adequate for the great majority of samples. In addition for tritium samples the ESR-efficiency curves are reasonably independent of volume as it is for chemically quenched carbon-14 samples. It is

imperative that individual curves be drawn up for particular volumes of colour quenched carbon-14 samples if sample sizes are to be varied.

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