

AN ADVANCED AUTOMATIC SAMPLE OXIDIZER - NEW
HORIZONS IN LIQUID SCINTILLATION SAMPLE
PREPARATION

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Abstract: Analysis of several animal tissues using the Packard Model 306 and two commercial tissue solubilizers (Soluenes) produced statistically identical results. Counting of tissue in an Insta-Gel H₂O suspension produced less counts per gram. The excellent performance of the Sample Oxidizer permitted: sequential analysis of labeled samples; dual analysis of ³H plus ¹⁴C singly labeled samples, with statistically accurate results. Sample preparation to final count may be achieved in 20-30 minutes. Tissue solubilizer digestions, to stable final count required 12-24 hours.

Introduction: It has long been the goal of the researcher using liquid scintillation counting techniques to reduce biological samples to a form which could be counted on a liquid scintillation spectrometer with zero quench. For tritium and carbon-14 labeled samples this is possible by converting water or carbon dioxide into labeled benzene or toluene, but it is not practical in a quantitative sense. Thus, based upon reality, the researcher is willing to compromise and settle for liquid scintillation counting cocktails giving a constant quench. One of the easiest ways to achieve this goal is to combust tritium and carbon-14 labeled samples producing tritiated water and carbon-14 dioxide. These materials are colorless and hence may be introduced into appropriate cocktails with only slight quenching appearing in the tritium

cocktail and a constant quench or a decrease in quench of the carbon-14 cocktail.

Historically, mass combustion of biological samples was initiated by Kelly *et al.*¹ using the Schöniger combustion technique². This combustion technique was further explored by Kalberer and Rutschmann³ and extended further to a microtechnique by Gupta⁴. Sample preparation with the macrotechnique was cumbersome and neither the macro nor microtechnique provided a physical means of separating tritium from carbon-14. It is well known that the liquid scintillation spectrometer was developed for separating instrumentally the carbon-14 beta energy appearing above the tritium spectrum from the tritium spectrum containing a portion of the carbon-14 spectrum. If adequate activity is present within a dual labeled tritium and carbon-14 sample, instrumental methods work quite well. If sample activity is low, adequate counting statistics cannot be collected within reasonable periods of time.

On the practical side, the researcher has long known the advantages of dual labeled techniques to eliminate the ever-present biological variation.

Kaartinen⁵ developed an experimental technique based upon a flowing oxygen flask combustion and gas scrubbing principle to provide physical separation of tritium as tritiated water from carbon-14 dioxide. This development was followed rapidly by the Peterson device⁶ which used catalytic combustion followed by a physical technique of separating the combustion by-products. These ideas led to the Packard 305 Sample Oxidizer and the Intertechnique Oxymat. Performance of these instruments was discussed at the 1970 International Conference on Organic Scintillators and Liquid Scintillation Counting, San Francisco, USA, by Sher *et al.*⁷ and Tyler *et al.*⁸ respectively.

The second generation Packard 306 Sample Oxidizer, offering simple pushbutton operation, has been examined critically and compared with

LIQUID SCINTILLATION COUNTING

tissue solubilizer digestion and suspension counting techniques. This study was undertaken to determine data quality; preparation effort; speed, from preparation to data availability.

Materials and Methods: Groups of four 160-180 gram female rats were treated by oral untubation with either 0.5 ml of [U-³H]-amino acid solution or 0.5 ml of [U-¹⁴C]-amino acid solution. The animals were allowed to remain undisturbed without food or water for four hours following treatment. The animals were anesthetized and sacrificed by decapitation. Tissues were removed directly from each carcass and placed immediately into a Dewar containing liquid nitrogen.

A third group of rats served as controls.

Samples were processed in the following manner. Dry ice was added to a Waring Blender jar while the motor was in operation until a powder was formed cooling the jar and powdered dry ice swirled around inside the blending jar. The liquid nitrogen was quickly decanted from the Dewar. The motor of the blender was turned off. The frozen tissue was added, the jar capped, and the blender turned to high speed. This technique reduced the frozen tissue to a fine powder. These tissue samples served as the source of all biological samples for subsequent data appearing in this report.

Biological samples were processed for liquid scintillation counting by three sample preparation techniques. These were combustion on the Packard Model 306 Sample Oxidizer, digestion in Soluene-100[®] or Soluene-350 and counting of the powdered tissue in Insta-Gel[®] water suspension.

Powdered animal tissue samples were weighed into tared Combusto-cones and burned in the Sample Oxidizer. The combustion timer was set for 1.2 minutes to permit both large and small samples to be processed at the same combustion time. The volumes of solutions used were: 15 ml of Monophase-40[™] added to the tritium collection vial and approximately 8 ml of Carbo-Sorb[™] (dial

setting 9) plus 12 ml of Permafluor[®]-V (dial setting 15) added to the carbon collection vial. A 0.35 ml aliquot of improved Combustaid[™] was added to each of the frozen tissue samples other than fat to initiate a rapid combustion. Fat samples were combusted in the presence of Whatman* cellulose powder to eliminate the possibility of any incomplete combustion (soot formation).

A 1 ml aliquot of Soluene-100 or Soluene-350 was added to each of a large group of liquid scintillation vials. The vials were capped with a polyethylene lined cap and tared. Aliquots of frozen tissue were added directly to the Soluene. The vial was reweighed to obtain a weight of tissue. Samples were digested both at room temperature with agitation, and at 50°C. Following complete digestion, 10 ml of toluene scintillator solution containing 4 grams PPO and 0.25 gram dimethyl POPOP per liter were added to each vial to complete the cocktail.

Other portions of tissue samples were weighed into a tared 10 ml aliquot of Insta-Gel. The vial was capped and the vial contents shaken vigorously. This disperses and wets the pulverized tissue with Insta-Gel. Four milliliters of water were added, the vial capped and shaken vigorously producing a suspension of particulate material in a rigid gel.

All samples were counted on a Packard Model 3390 Tri-Carb Liquid Scintillation Spectrometer. The tritium was counted in a wide window (³H pushbutton). The carbon labeled samples were counted in a wide window (¹⁴C pushbutton).

Results and Discussion: The collection of quality liquid scintillation data from biological samples depends upon having an adequate knowledge of various parameters of the liquid scintillation cocktail and sample. Various animal tissues have

*Whatman is the registered trademark of W&R Balston Ltd., Maidstone, Kent, England.

LIQUID SCINTILLATION COUNTING

been subjected to combustion using the Sample Oxidizer. An analysis of both the tritium and carbon-14 backgrounds demonstrates the background of the resulting cocktails is independent of tissue weight over the examined range of 0 to 0.6 grams. Tissues examined were beef muscle, pork liver, and beef fat (0 to 0.2 grams).

The rat tissue samples of control animals were examined in the same way. Liver control samples indicated a tritium content of 219 ± 18 DPM/gram of tissue with weights ranging from 0.1 to 0.58 gram. Rat muscle control exhibited a background of 176 ± 28 DPM/gram. The rats were maintained in a laboratory where we store trace quantities of tritiated water and tritiated toluene. It now became apparent that our control animals and presumably our treated animals contained trace amounts of tritium within the tissue. This sensitivity of tritium detection points out one of the advantages of the Sample Oxidizer.

A second factor which must be evaluated in rapid processing of biological samples is the length of time required to produce the sample and then the length of time required to achieve constant counting conditions. The analytical weighing time to prepare the above samples is nearly identical for each of the three procedures of sample preparation since two weighings are required for the production of each sample. Both combustion and suspension of tissue samples in Insta-Gel water are very rapid. Digestion in Soluene-100 or Soluene-350 requires approximately two hours for 0.1 to 0.12 grams of tissue at 50° C and overnight digestion with periodic agitation is required for these same reagents at room temperature.

The Model 306 Sample Oxidizer tritium cocktail described above reached a background equilibration in approximately six minutes. Following that point in time, only one observation out of 24 fell outside the two standard deviations. This represents a rapid stability of background by placing a room temperature cocktail into a subambient temperature-controlled Tri-Carb

Scintillation Spectrometer. A tritium sample combusted utilizing the Sample Oxidizer falls within expected counting statistics at the same period in time. Thus, tritium samples may be processed on a Sample Oxidizer with adequate counting data some six to 10 minutes following actual combustion of the sample.

A background sample derived from the carbon side of the Sample Oxidizer was constant for one minute counts from the point of time where it was introduced into the Tri-Carb. This cocktail contains Carbo-Sorb (an organic amine) as a trapping agent. Since amines are strong quenchers to the liquid scintillation process, the quenching of a carbon sample should decrease until the sample reaches temperature stabilization. Previous experiments in our laboratory had demonstrated the minimum time to achieve this temperature stabilization was approximately 45 minutes. To investigate this 45 minute wait, a carbon sample was burned on the Sample Oxidizer and counted continuously using one minute counts until stabilization was achieved. The stabilization occurred at 15 minutes.

These count rate stabilization times for the Sample Oxidizer cocktails are very significant since samples may be counted 15 minutes following sample preparation. This permits the investigator to obtain meaningful data and make necessary corrections in critical experimental projects. In the past this has not been possible.

A Soluene-100 or Soluene-350 plasma sample exhibits a reasonable degree of chemiluminescence. Initial counts of 3,000 CPM are quite common. These drop off quite rapidly in an exponential manner. In our experiment the background dropped below 100 CPM at approximately 30 minutes after the sample was introduced into the liquid scintillation spectrometer. For high specific activity samples a 100 CPM background may require one or two days for total stabilization of the background counts. This background is normally attributed to chemiluminescence but exists in the carbon-14 channel as well as the

LIQUID SCINTILLATION COUNTING

tritium channel. Duplicate samples prepared from the same reagents may produce chemiluminescence in one vial but not the second. Thus, additional time is required to be sure that labeled samples have definitely stabilized.

Insta-Gel water suspension of pulverized tissues achieves a stable background within five minutes. Since quantitative data was not achieved with this technique, temperature stabilization times for emulsion of radioactive tissue samples were not measured.

The various rat tissues from single labeled samples, either tritium or carbon-14, were burned on a Sample Oxidizer and the activity obtained converted to a specific activity expressed as DPM/gram. This data is presented in Table I. Most of the labeling is about as one would expect following oral administration of a labeled material. It may be interesting to note that the fat samples derived from pooled omental and renal fat were quite high in terms of apparent labeled activity.

The standard deviations of the tissues range from very low to moderately low. As one might predict, the gastrointestinal tract variation is higher than others. This is certainly a very difficult area to sample and more than one animal is represented in the sample. The standard deviation of the data appears to have some correlation with the ability to thoroughly pulverize the sample. In samples which contained chunks of the original tissue, the standard deviation generally exceeded 2%. In samples which had no chunks remaining within the tissue, the standard deviation was generally 2% or less. In one case, for the tritium brain tissue, the standard deviation was 0.25%. This is indeed excellent reproducibility for tissues. Tissue weights range from approximately 40 mg to 400 plus mg.

Most investigators will probably concede that a Sample Oxidizer is capable of producing quality data. A knowledge of how the results of experimental data of this technique compared with

tissue solubilizers and a third technique, suspension in a gel emulsion, allows the investigator to evaluate the possible techniques. A comparison of this data is presented in Table II. Careful examination of the data in Table II demonstrates very significantly that the two Soluene tissue solubilizers give data equal to the 306 Oxidizer.

In only one case the Soluene appeared to give a higher specific activity than the Sample Oxidizer. This is for carbon-14 labeled rat muscle. As stated previously, it was demonstrated that the control rats sacrificed some days before the treated animals contained traces of tritium contamination. The data obtained from the Sample Oxidizer would contain no tritium in the carbon-14 vial. A statistical analysis of a wide group of carbon-14 tissues combusted demonstrated one count per minute less for the possible tritium spill into the carbon-14 vial than was observed in a composite of blanks containing no tissue material. Thus, the 306 Oxidizer sample would contain no tritium whereas the Soluene-100 and Soluene-350 samples would definitely contain the tritium as well as the carbon-14 present within the sample. This higher value does not occur in the carbon-14 labeled rat gastrointestinal tract. On further examination it appears that the Sample Oxidizer exhibits a lower percent standard deviation than do the tissue solubilizers.

Insta-Gel water suspension gives less than the theoretical amount of tritium and carbon activity. The rat muscle suspensions, which create only slight quenching, are very reproducible using this technique. However, this strongly demonstrates that reproducibility does not mean correct data. The tissue samples were simply pulverized in the presence of powdered dry ice. The technique might still offer considerable promise if the particle size could be further reduced.

The specifications for the Model 306 Sample Oxidizer (both tritium and carbon-14 recoveries

LIQUID SCINTILLATION COUNTING

are $99 \pm 1\%$; tritium memories and carbon-14 memories less than 0.05% ; carbon-14 residual remaining in the tritium vial of less than 0.02% ; and tritium spillover into the carbon-14 vial of less than 0.001%) suggest many unique things can be done with the Sample Oxidizer. In the past, due to the presence of reasonable memories, spillover, and residual activities, it has been desirable to burn blank samples between each radioactive sample which is combusted. Therefore, based upon the above specifications, an experiment was devised to burn labeled samples, containing single label and widely varying weights, one after another with no blank combustion between the samples. All data would be calculated to DPM/gram. Thus, a large sample followed by a small sample should produce a higher than expected DPM/gram for the small sample. The data from this experiment is included in Table III. Examination of the tritium data column demonstrates a 0.414 gram sample on line 2 followed by a 0.060 gram sample. The lesser sample does not have a higher specific activity. In the same column there is a 0.485 followed by a 0.111 sample. Likewise, a 0.239 followed by a 0.095. Neither of these latter samples has a higher specific activity than the preceding sample. A similar discussion may be presented for the carbon-14 data column. In the case of the 0.463 followed by the 0.079 there does indeed appear to be an increase. However, this value falls very close to the two sigma limit of the average. More significantly, the pile-up of memories should yield a larger average DPM/g for samples burned one after another than should a series of samples burned with blanks between each sample. Therefore, a group of samples was burned along with blanks between each sample. The tritium specific activity from the rat muscle was $186,500 \pm 2,930$ (1.57%) DPM/gram. The carbon-14 specific activity was $15,140 \pm 320$ (2.11%) DPM/gram. These values are experimentally identical to the values obtained by burning samples one

after another.

Knowing that it is possible to burn samples one after another, with insufficient cross-talk or memory to produce a statistical difference in the results, it was theorized that singly labeled tritium and carbon-14 samples could be burned in combination to produce DPM data for each of the single labeled isotopes. In principle, this is the only way the Sample Oxidizer may be checked to determine adequate performance from labeled biological tissues. Thus, singly labeled tritium samples were weighed into Combusto-cones. Carbon-14 labeled samples were weighed into a second Combusto-cone. The two cones were stacked one within the other, Combustaid was added, and the samples were burned. The weight of tritium sample to carbon-14 sample was varied widely.

In various weight ratios once again all data was calculated to a specific activity of DPM/gram. This data is presented in Table IV. An analysis of the data indicates the combustion technique using the Model 306 Oxidizer is independent of sample size and produces constant results for tritium activity and carbon-14 activity for widely varying combinations of sample weights. This technique thus provides a means for analyzing single labeled samples in tritium-carbon combinations to yield DPM per unit weight or volume with a high degree of accuracy. Sample preparation time is rapid and results consistent.

A comparison of the data obtained by burning samples alone with no blanks between samples; by burning samples alone with a blank between each sample; and burning samples in combination (tritium and carbon-14 being burned simultaneously) gives statistically identical results.

The Model 305 manufactured by Packard, the Oxymat (Reich modification of Peterson system), and the Harvey oxidizer have significant memories and spillover. These spillovers and memory would likely preclude any dual combustion of singly labeled tritium and carbon-14

LIQUID SCINTILLATION COUNTING

samples or sequential combustion of labeled samples with no blanks between. Thus, the Packard Model 306 Sample Oxidizer does indeed open up new horizons in liquid scintillation sample preparation with wide flexibility, easy sample preparation, and quick accumulation of accurate counting data.

References

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TABLE I

Specific Activity (DPM/gram) of Rat Tissues Separately Labeled with Either [^3H] or [^{14}C] - Amino Acids (Sacrifice Four Hours After Oral Intubation)

<u>Rat Tissue</u>	<u>^3H ($\times 10^3$)</u>	<u>^{14}C ($\times 10^2$)</u>
Brain	193.5 \pm 0.5 0.25%	166.0 \pm 3.6 2.19%
Fat	219.7 \pm 4.4 2.01%	463.0 \pm 31.0 6.72%
GI Tract	559.5 \pm 43.1 7.71%	1190.0 \pm 26.0 2.18%
Heart	231.5 \pm 6.2 2.68%	337.0 \pm 3.4 1.00%
Kidney	351.0 \pm 19.5 5.55%	749.0 \pm 12.7 1.69%
Liver	491.7 \pm 7.5 1.53%	_____*
Muscle	195.5 \pm 2.4 1.24%	150.0 \pm 1.1 0.74%
Spleen	321.7 \pm 6.4 2.00%	777.0 \pm 29.3 3.77%

*Sample was not completely ground; large chunks of liver made sampling impossible.

TABLE II

A Comparison of Calculated Specific Activity (DPM/gram) of Rat
Tissues Using Different Sample Preparation Techniques

Sample Preparation	Rat Muscle		Rat GI Tract	
	^3H ($\times 10^3$)	^{14}C ($\times 10^2$)	^3H ($\times 10^3$)	^{14}C ($\times 10^2$)
306 Oxidizer	195.5±2.4 1.24%*	150.0±1.1 0.74%	559.5±43.1 7.71%	1190±26 2.18%
Solvene-100	195.7±6.2 3.15%	165.0±13.6 8.27%	560.2±57.3 10.2%	1169±74 6.35%
Solvene-350	197.0±5.7 2.88%	160.0±3.7 2.28%	571.5±37.6 6.58%	1217±54 4.45%
Insta-Gel--H ₂ O	150.0±3.0 2.01%	103.6±3.6 3.76%	279.2±33.6 12.0%	772±116 16.2%

*Percent Standard Deviation was calculated from data before rounding off; direct data manipulation may yield a slightly different value.

TABLE III
Constant Specific Activity from Single Labeled
Animal Tissue with No Blank Combustion Between
Samples

Tritium Rat Muscle		Carbon-14 Rat Muscle	
Sample Wt. (grams)	Activity DPM/gram	Sample Wt. (grams)	Activity DPM/gram
0.1112	186,000	0.0847	15,130
0.4145	191,800	0.2798	14,880
0.0600	180,900	0.1086	15,270
0.3019	183,000	0.4633	14,820
0.0839	183,800	0.0798	15,950
0.2890	187,100	0.2749	15,030
0.4855	189,700	0.1829	15,320
0.1118	182,100	0.0815	14,880
0.2399	187,400	0.1417	14,950
0.0959	184,100	0.2264	15,350
Average	185,600		15,160
Std. Dev.	3,500		340
% Std. Dev.	1.89		2.24

TABLE IV
Analysis of Singly Labeled Tritium and Carbon-
14 Samples by Combustion of Both Samples
Simultaneously

Tritium Rat Muscle		Carbon-14 Rat Muscle	
Sample Wt. (grams)	Activity DPM/gram	Sample Wt. (grams)	Activity DPM/gram
0.0494	187,200	0.1978	15,100
0.3295	186,900	0.0698	15,180
0.2893	189,800	0.2436	14,900
0.2698	189,700	0.1804	14,850
0.2153	187,400	0.0655	15,590
0.0777	188,300	0.3368	15,290
0.0400	180,500	0.0819	15,170
0.4103	188,200	0.2244	14,940
0.0755	184,400	0.0902	15,240
0.0523	189,100	0.4266	14,750
Average	187,200		15,100
Std. Dev.	2,820		250
% Std. Dev.	1.51		1.66