

A Comparison of Combustion Techniques Used in the Preparation of ^{14}C -labelled Samples for Liquid Scintillation Counting from Biological Materials

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INTRODUCTION

The preparation of homogeneous samples for the radioassay of β -emitters in a wide range of biological materials is a problem which has almost as many answers as there are analysts and opinions. It is, however, generally accepted that, for labelled tissues, combustion is the most accurate and frequently the most convenient method. This chapter confines itself to the author's personal experience of combustion techniques and of their application to samples labelled with Carbon-14 only. The more important of the methods I have tried are outlined with brief descriptions of the apparatus and the results obtained. A comparison is then given of the methods in current use in our laboratories, particularly with respect to reliability in the multi-user situation.

THE PEETS AND FLORINI TUBE FURNACE

The method developed by Peets and Florini (Lederle Laboratories, American Cyanamid 1960)¹ is of current interest in that a low-cost biological oxidiser based on the same principles is now marketed by Beckman and the Harvey Instrument Corporation. The apparatus is an enlarged and modified version of the standard carbon/hydrogen microanalysis furnace. It can oxidise samples of up to 2 g dry weight. The filling of the silica tubes consists of a loose roll of acid-washed nickel foil about 20 cm long and a 2 cm thick pad of loosely matted silver wool. Three such tubes are mounted in an electrically heated furnace. The catalyst section is maintained at about 950°C, the sample section is gradually heated to 750°C. Current to the heating elements is controlled by a timer and switching device to give an adjustable rate of temperature increase. The Lederle group quote Pregl and Grant in claiming this type of filling to be as efficient as the conventional copper oxide filling when used as a combustion catalyst, and also claim that it has no tendency to pack and retard gas flow as does copper oxide. Water of combustion is collected in a borosilicate glass trap immersed in a slurry of dry ice and chloroform. Carbon dioxide is collected in a spiral containing 10 ml of 5N sodium

hydroxide which has a maximum capacity equivalent to 300 mg carbon. This undesirable absorbent has since been replaced by a cocktail containing an organic amine.

Using the original apparatus we found the recommended cycle time of 45 min led to explosions with some samples; in fact, for some fatty materials, double the suggested cycle time was more realistic. The surface of the nickel catalyst was attacked and a build-up of material blocked the interstices of the nickel roll. Recovery was determined both chemically and radiochemically as a little over 90%. The losses, which were in the trapping of the carbon dioxide, could be accounted for when a second absorption spiral was introduced in the train. We used the technique exclusively for very low activity samples and 'memory effect' proved no problem. The Beckman/Harvey instrument, which is claimed to process eight samples an hour, is being used by our colleagues at I.C.I. America. Their reports have been good but further comment would be unwise before trying the instrument ourselves.

THE KALBERER AND RUTSCHMANN OXYGEN FLASK (Fig. 1)

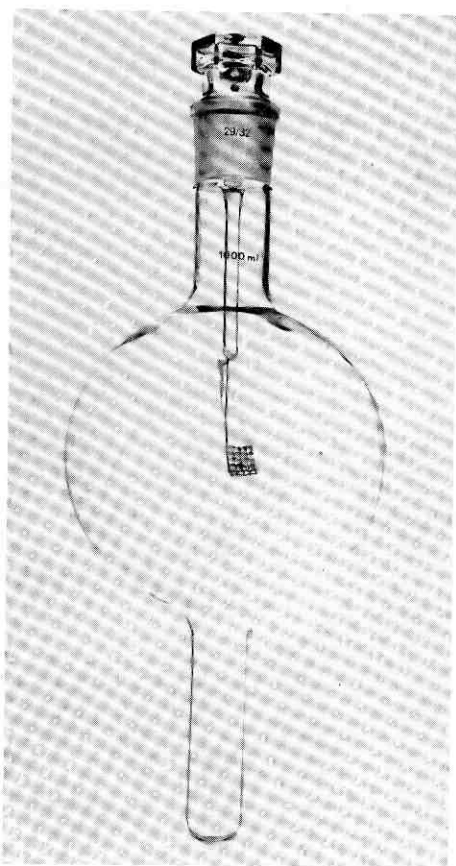


Fig. 1. Photograph of Kalberer-Rutschmann flask

As in the classical Schöniger technique for elemental analysis, organic material is burned in pure oxygen in a closed vessel using a platinum support. There are many excellent techniques based on this approach, including that of Dobbs.³ The

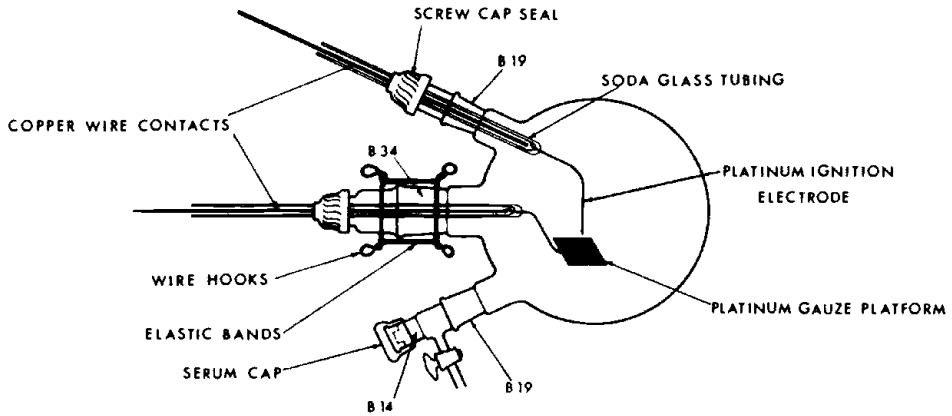


Fig. 2. The Dobbs flask

Dobbs flask (Fig. 2) will cope with larger samples than the Kalberer–Rutschmann flask (Fig. 3). Impressive results were obtained in our laboratory. However, for various, including logistic, reasons we continued with the Kalberer–Rutschmann

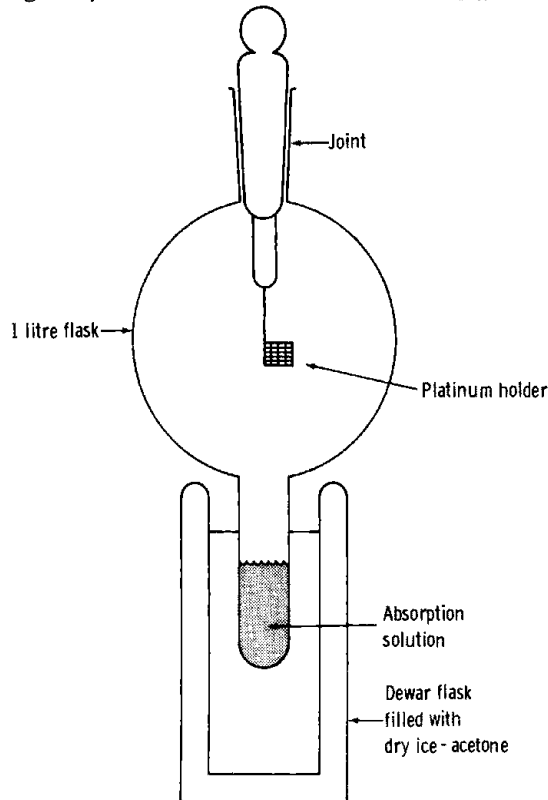


Fig. 3. Diagram of Kalberer–Rutschmann flask

approach in which the absorption medium (12% ethanolamine in methanol) is contained in a finger-like extension to the combustion flask which is cooled in dry ice-acetone contained in a Dewar vessel. This lowers the vapour pressure of the methanol and prevents the formation of an explosive solvent-oxygen mixture in the flask. This process is normally carried out in a shielded stand which can be rotated electrically to ensure efficient absorption of combustion products. 0.5 ml blood or up to 400 mg tissue is taken and the sample is air dried. The absorption solution is introduced into the cold finger and the flask is then flushed with oxygen. The paper tail is ignited. Combustion is rapid and safe. The flask, normally in the special stand, is rotated for 20 min to facilitate absorption of the combustion products. An aliquot of the absorption solution is added to butyl PBD in toluene for scintillation counting. Recoveries are good but, as in all such techniques, should be checked using samples spiked with the radio-labelled compound of interest at a level relevant to the problem in hand. Table 1 shows such a series at low levels of activity. Memory effect is non-existent with a well organised system for cleaning the apparatus.

Table 1. Recovery of radioactivity added to control muscle tissues.

Added ¹⁴ C (d.p.m.)	100-min count	Δ c.p.m. ^a	Corrected ^a for efficiency and sampling	% Recovery
4.9	3,003	3.03	4.9	100
4.9	3,031	3.3	5.4	110
9.8	3,357	6.6	10.7	110
49.1	5,006	23.1	37.7	76.7
49.1	5,767	30.7	50.0	102
98.2	8,688	59.9	97.6	99.5
98.2	8,398	56.4	92.0	94.0
147.3	10,988	82.9	135.0	91.5
147.3	11,117	84.2	137	93.0
196.4	13,851	111.5	182	92.5
196.4	13,822	111.2	181	92.0
245.5	16,965	142.5	232	94.5
245.5	16,713	140.1	229	93.0
				$\bar{x} = 96.1$
				$s = 8.7(9.0\%)$

Packard Tricarb Series 3003 used.

^a Average background 27.0 c.p.m. and average counting efficiency of 68.0% used in these calculations.

THE PACKARD 305 SAMPLE OXIDISER (Fig. 4)

The sample is placed in the Platinum-Rhodium ignition basket which is held in place by a pneumatic piston. Combustion is controlled by operator regulation of the oxygen flow and basket heater current. Water from the combustion is trapped in a condenser at 2°C and caught in the counting vial under the condenser. Carbon dioxide and other gases pass through the condenser and vial to a CO₂ reaction column filled with ethanolamine. This column is automatically washed with scintillator and the products recovered directly in a counting vial. The system is flushed with nitrogen after the combustion.

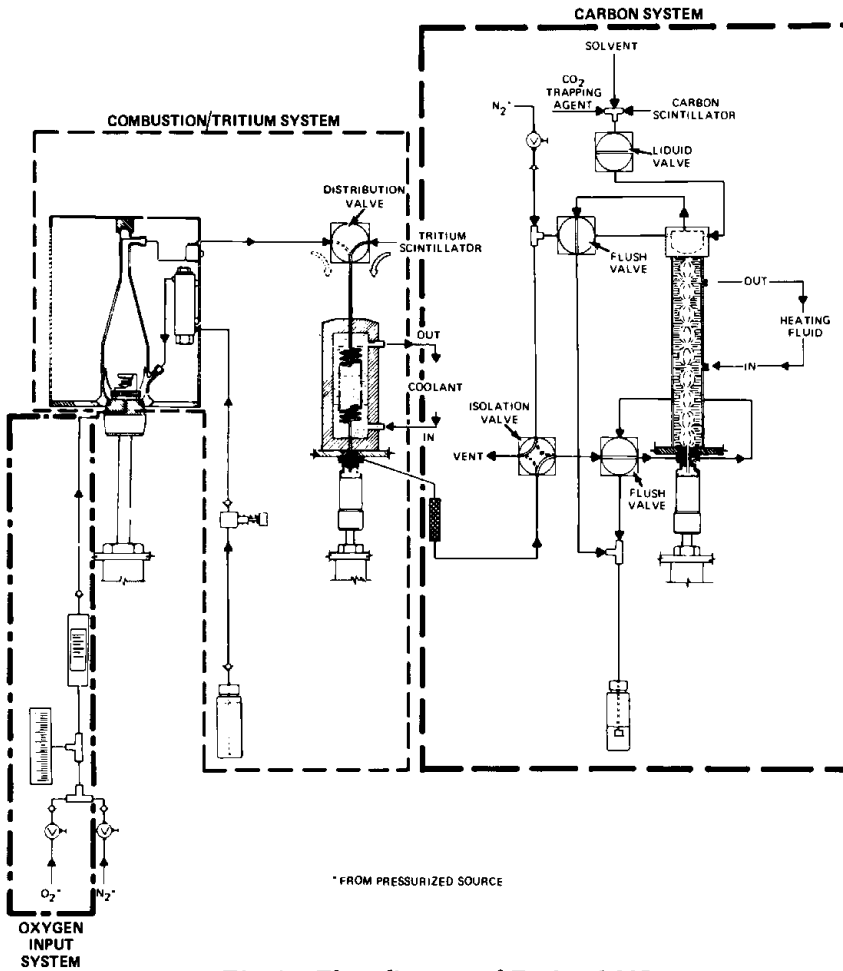


Fig.4. Flow diagram of Packard 305

A Packard 305 has been in our laboratory for 2½ years and used routinely by our metabolic groups for a little over one year. A considerable number of mechanical difficulties were experienced and enthusiasm waned as it became apparent that both time and care had to be lavished on the instrument to maintain a good level of performance. Usage of the instrument dwindled and stopped completely before the end of the second year. At best, recovery and memory were up to the manufacturer's specification, but as Dr. Scales indicates elsewhere (p.211), an accumulation of errors can lead to poor recoveries before the relevant faults in the instrument are obvious to the casual user. The major difficulties were caused by blockage and leakage in various lines and valves. A retrospective examination of routine results (Table 2) showed that when the Packard 305 was working well the assays were as reproducible as those produced by the flask combustion technique.

THE INTERTECHNIQUE OXYMAT SAMPLE OXIDISER (Fig. 5)

In this instrument, which is based on a system devised by Peterson (National Institute of Health), combustion takes place in a stream of pure oxygen. The sample, in a small polycarbonate sachet, is introduced by an electromechanical

Table 2. Retrospective comparison of Packard 305 and Kalberer—Rutschmann flask.

Percentage standard deviation for d.p.m./ml blood		
Range (d.p.m. $^{14}\text{C}/\text{ml}$)	100—1,000	1,000—10,000
K.R. flask	7.9%	5.6%
Packard 305	5.9%	3.7%

Each value represents 15 samples taken at random from the results of about 10 operators.

system into a furnace heated to 700°C . A catalyst completes the oxidation. The combustion products pass through a heated transfer line and water and carbon dioxide are separated and trapped by two cooled, wiped film centrifugal absorbers. The descending film of trapping fluid is met by the countercurrent stream of combustion products. The combination of a thin liquid film and the turbulence impressed on the gas ensures rapid and efficient trapping.

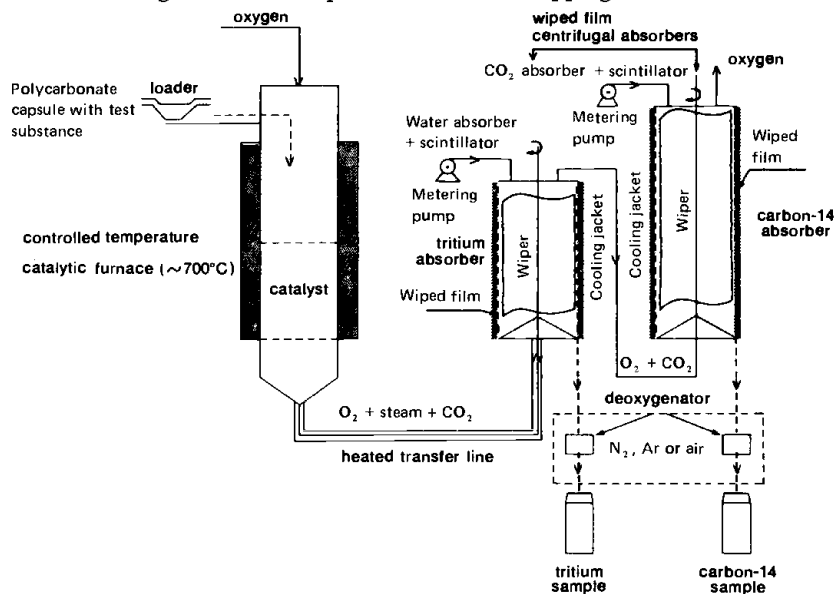


Fig. 5. Flow diagram of the Intertechnique Oxymat

In the first absorber, water vapour is condensed and absorbed in a dioxane—methanol—water phosphor system. Carbon dioxide is trapped in the second absorber using a toluene—phenylethylamine—methanol—water phosphor system. As the liquid flows into its respective counting vial it is de-oxygenated by purging with a suitable gas such as nitrogen.

The first of three attempts to evaluate the instrument was in early 1972. After less than 100 samples a large amount of carbon was deposited throughout the system and massive temperature fluctuations were noted in the furnace. The manufacturers were informed and later modified the combustion tube design,

holding back the solid particles with a bed of silica sand covered by coarse carborundum chippings. They also uprated the furnace.

In the second attempt at evaluation (June 1972), good recoveries (>98%) and low memory (<0.1%) were obtained with small dry samples. However, after 70 samples were burned in rapid succession, recovery dropped rapidly to 60-70%. On the second day only 30 samples were combusted successfully before large losses were noted. This was later attributed by the manufacturers to leakage at the seal of the drive shaft to the first absorber. At our request a series of 100 small dry cellulose samples were combusted by the manufacturers on yet another instrument. The recoveries were good but, since this was only a fraction of the required testing indicated by our previous experience, and took six months to accomplish, we were not yet entirely convinced.

Because of our requirement for a rapid instrumental solution to our problem we purchased an Oxymat in June 1973 subject to certain stipulations regarding performance. There were assembly faults which we were able to adjust ourselves. Only a modest number of results can be reported since, unfortunately, at the most interesting point in our investigations the solenoid controlling the sample entry port self-destructed. Seven of our staff each performed a series of combustions as indicated in Table 3. In addition they combusted an equal number of non-radioactive samples which indicated that memory was less than 0.4%. Recovery was excellent and similar to that obtained by the manufacturers in the experiment

Table 3. Operation of the Oxymat. Recovery of added ^{14}C from blood, muscle (minced and part dried) and cellulose.

Added ^{14}C	Percentage recovery			Mean
	Blood 0.5 ml + 500 mg cellulose	Muscle ~400 mg	Cellulose ~500 mg	
10,000 d.p.m.	94.86	98.87	92.51	95.41
500 d.p.m.	97.56	102.77	98.67	99.67
Mean	96.21	100.82	95.59	97.54

Average standard deviation = 2.5%.

Each value is the average result obtained by seven operators.

mentioned previously. We decided to investigate losses we observed when trying to combust wetter tissue and moistened cellulose samples. Using the simplest of traps, to avoid introducing back pressures, the effluent gases were shown to contain carbon dioxide including a high proportion of the Carbon-14 added to the samples. I was not surprised to note that the luminous stage of some of these combustions was not completed until most of the absorbent was already in the vial. We did not, at this stage, wish to take a hacksaw to the timer cams in order to prolong the absorption step but we did improve recoveries by introducing 60 Hz mains cogs into the timer drive (Fig. 6). This prolonged all stages controlled by the timer, and of course necessitated adjustment of the absorbent pump flow rates. We have only one series of results, for the reasons mentioned earlier, but these do show a trend worthy of further investigation. It appears in fact to bring the instrument up to the manufacturers specification, for these more awkward samples.

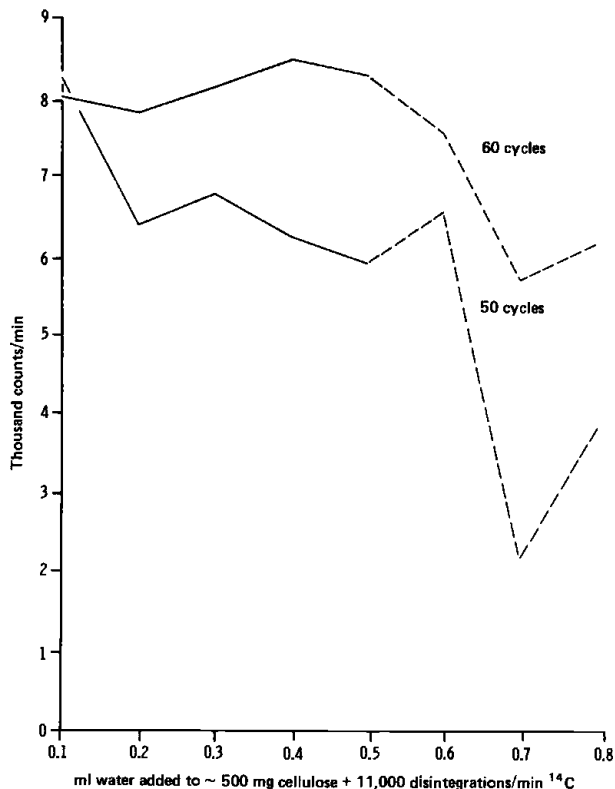


Fig. 6. Effect of 60Hz drive wheels on the performance of the Oxy-mat

THE PACKARD 306 SAMPLE OXIDISER

The appearance of the model 306 confirms its description as a 'second generation' model. Although the basic layout is similar to the model 305, there has been considerable streamlining to eliminate the less reliable features of the earlier model. As the flow diagram (Fig. 7) indicates, the principle remains the same as the model 305, a single push button initiates the positioning of the vials and combustion basket, combustion, injection of liquid scintillation cocktails, unloading and a cleaning cycle. After the first 20 samples we noted a reduction in the delivered volume. This was traced to the metering syringes, which were cleaned and re-assembled by the Packard engineers, but a recurrence of the fault necessitated replacement of the parts. After a few more samples, losses in volume were noted which were traced to a blockage at the top of the column obstructing the flow of the absorbent. Packard were aware of this fault which they believe they have now eliminated. The column was, however, easy to strip and clean.

Recovery of added Carbon-14 from cellulose, plasma and fat was quite good but losses were noted when moist muscle was burned (Table 4). In fact, recovery appeared, to some extent, inversely proportional to the required combustion time. Packard recommend the use of combustion aids with some samples and it is perhaps unfair not to have followed their advice, but this was intended as a preliminary investigation to acquire the 'feel' of the instrument. Memory was impressively low at less than 0.03%.

An additional series of cellulose samples with 50 d.p.m. Carbon-14 added and part dried muscle with 500 d.p.m. Carbon-14 added gave recoveries around 95%. At this point we thought that we could settle down to a more serious evaluation of

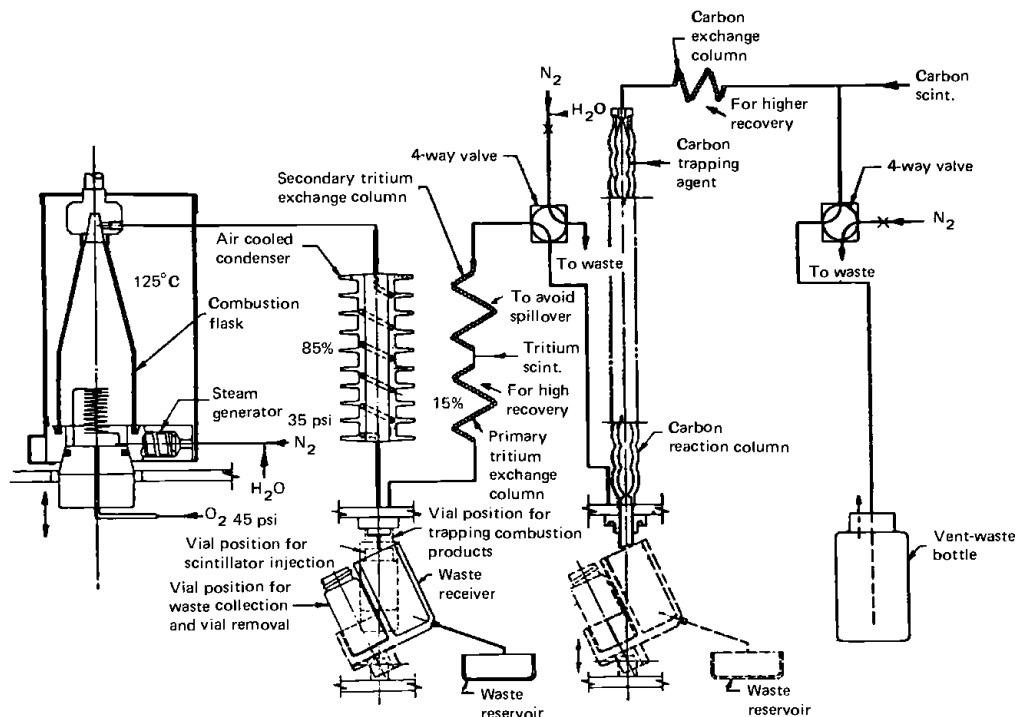


Fig. 7. Flow diagram of Packard 306

the instrument. Unfortunately, we had further recurrences of the liquid dispensing problem. We were informed by Packard that this was due to the high ambient temperatures in our laboratories during a recent spell of very hot weather. This

Table 4. Operation of the Packard 306. Recovery of added ^{14}C from cellulose, plasma, fat and muscle (minced and part dried); 10,000 d.p.m. ^{14}C added to each sample.

Sample	Percentage recovery
Cellulose 500 mg	100.1
Plasma 0.5 ml + 350 mg cellulose	92.1
Fat 250 mg + 250 mg cellulose	90.3
Muscle 500 mg	72.7

Average standard deviation = 5.4%.

Each value is the average of 10 analyses.

fault had not occurred in the instrument prototype which was developed in air-conditioned laboratories. We were told that modified seals were now being fitted which had overcome this problem. We can only look forward to evaluating a

modified instrument at the earliest opportunity.

CONCLUSIONS

The oxygen flask technique stands out for its reliability. Bearing in mind the time wasted on breakdowns and maintenance of commercially available equipment, manual techniques can often be quicker and cheaper in the long run. In fact, if an equal financial investment were made in support equipment for a flask combustion set-up it would require a very good instrument to better it. The Packard 305 can be regarded as a first venture into instrumental methods and we have learned a great deal from it. The Packard 306 appears promising if the one major fault so far detected has now been overcome. The Oxymat impressed us as an elegant concept but the manufacturers were a little slow to make the final adjustments to design which would bring its performance up to specification. It is, however, an instrument which the user can adapt to suit his own requirements.

ACKNOWLEDGEMENTS

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DISCUSSION

P. Johnson: You mentioned early in your talk that it is desirable — in fact essential — to check combustion recoveries by spiking with the compound itself which is under investigation. In other words, the standard should be closely related structurally to the test sample. This in my opinion is one of the problems with combustion methods. With a solubiliser method, as I pointed out earlier, one has to prove that there is no loss of volatile materials but this does not require any knowledge of the labelled components — it either happens or it doesn't — and in any case can be prevented by using vials sealed with septa. In combusting biological samples, however, we are often dealing with mixtures of unknown labelled components; therefore, how can one choose a structurally related compound for spiking? If recoveries were not dependent on structure, then one would of course be free to standardise with anything; in fact, many people do so, and risk the probably small number of times that it will cause trouble.

R.G. Cooper: It is probably not essential to choose a standard which is structurally similar to the compound under investigation, and as you say it is often impossible, but we do compromise by using the parent compound in a metabolic investigation.

P. Johnson: But you don't identify the metabolites and use them as standards.

R.G. Cooper: We have on occasions, but not routinely.

M. Stiasni: You showed that you combusted 0.5 ml blood + 500 mg cellulose. This is a remarkably big amount and disagrees with Dr Gordon's paper in which the recovery drops down with sample weights more than 200 mg.

R.G. Cooper: Please ask Dr. Gordon. I don't remember the exact composition of his absorption solution, which may, of course, be highly relevant.

B.E. Gordon: Our absorption solution contains about 27% v/v of phenethylamine in a cocktail of methanol plus toluene plus the usual fluorescers. The volume of absorbing solution is sufficient to neutralise about 18 mmoles of CO₂ or 216 mg of carbon. This value is not reached because before all the phenethylamine is quantitatively converted to the carbamate, precipitation occurs (the trap is chilled with ice) and CO₂ is no longer quantitatively absorbed. Use of a cocktail with a better solvent power for the carbamate, use of more absorbing solution plus a counting vial of greater capacity than those currently in vogue would increase the amount of CO₂ which could be trapped. The bottleneck is not in the burning but in the trapping.

N. Kaartinen: What is the capacity of the Packard 306 CO₂ trapping agent?

R.G. Cooper: 40 mmoles CO₂/13 ml Carbosorb, including the margin in the degree of saturation. A remark concerning the low blood recovery. When wet samples are combusted without Combustaid, and thus without a flame, pyrolysis products escape through the exhaust. A sufficient amount of Combustaid should be used.