

## AUTOMATED ANALYSIS OF $^{15}\text{N}$ AND $^{14}\text{C}$ IN BIOLOGICAL SAMPLES

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**ABSTRACT:** An automated method for the simultaneous analysis of total N, total C,  $^{15}\text{N}$  and  $^{14}\text{C}$  in small plant and soil samples is described. A commercial C-N analyser - continuous flow isotope ratio mass spectrometer (ANCA-MS) has been extended to also measure  $\text{CO}_2$  and collect  $^{14}\text{CO}_2$  produced by sample combustion. Samples containing 20 - 200  $\mu\text{g}$  N and up to 5 mg C can be analysed directly with no sample preparation other than drying and fine grinding. The precision of total elemental analysis is comparable to that by conventional methods. The average standard deviation of  $^{15}\text{N}$  analyses of plant material at natural abundance was  $\pm 1$  ‰. This is accurate enough for all  $^{15}\text{N}$  studies except those using natural abundance and possibly long term studies of soil organic matter. Recovery of  $^{14}\text{C}$  in test samples was 100%. The instrument can be operated by graduate students under supervision and operating costs are primarily for sample cups, combustion catalyst and quartz tubes.

## INTRODUCTION

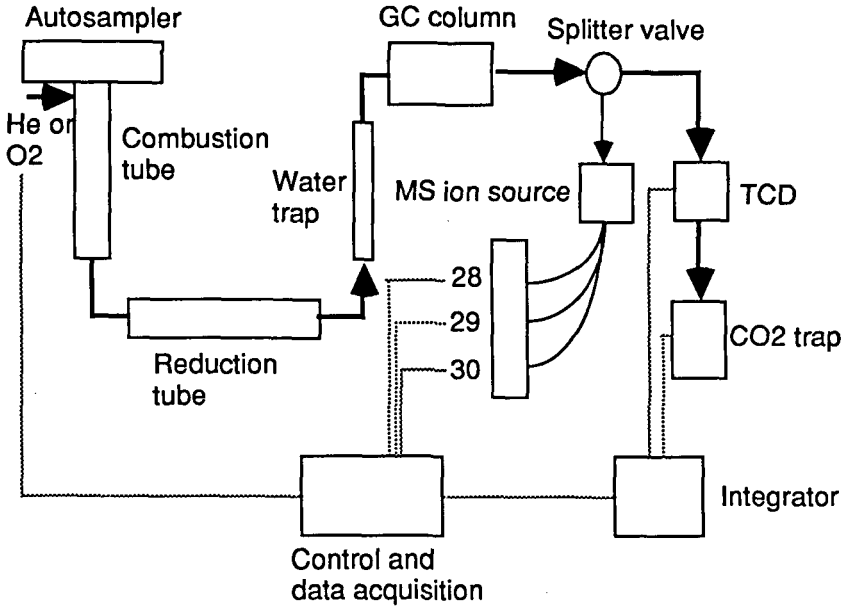
The biological transformations of carbon (C) and nitrogen (N) are closely linked and it is therefore useful to use simultaneous tracers for both elements in studies of diverse biological processes. The use of  $^{15}\text{N}$  tracers in particular has been limited by the expense and difficulty of sample preparation and analysis by the Kjeldahl-Rittenberg procedure (1). Solid samples containing  $^{14}\text{C}$  are commonly analysed by three main methods: a) wet oxidation (2,3) followed by trapping of  $\text{CO}_2$  in base and analysis of total C by titration and  $^{14}\text{C}$  by scintillation counting, b) combustion and trapping of  $\text{CO}_2$  in a  $\text{CO}_2$ -absorbing scintillation cocktail, and c) solubilization of the sample in a medium such as methylbenzethonium hydroxide which can be mixed with a suitable scintillant (4).

The commercial availability of an automated instrument combining in-line sample combustion for conversion of sample N and C to  $\text{N}_2$  and  $\text{CO}_2$ , and continuous flow isotope ratio mass spectrometry (Automatic Nitrogen and Carbon Analyser - Mass Spectrometer, ANCA-MS) (5) greatly facilitates the use of stable isotopes of C and N as tracers. The instrument is designed for automated analysis of either  $^{15}\text{N}$  or  $^{13}\text{C}$ , but the isotopes cannot yet be analysed simultaneously.

The major portion of the sample combustion products are not required for mass spectrometry and are normally vented to waste. We have extended the system by adding a thermal conductivity detector (TCD) for measurement of total  $\text{CO}_2$  and automated trapping of  $^{14}\text{CO}_2$  in scintillation cocktail (Fig. 1). Samples containing 20 to 200  $\mu\text{g}$  of N and up to 5 mg C can be analysed directly for total N,  $^{15}\text{N}$ , total C and  $^{14}\text{C}$  with no preparation other than drying and fine grinding. Analysis time is 7 minutes per sample, 100 samples can be processed in day and overnight runs each of 50 samples plus 14 references.

## METHODS

**SAMPLE PREPARATION:** Plant and soil samples were dried at  $60^\circ\text{C}$  and ground to  $< 250\ \mu\text{m}$  in glass jars (Qorpak # 2143, Fisher Scientific) containing 4 - 6 stainless steel bars (3 x 50 mm). The jars were rotated for 5 - 24 h on a roller mill consisting of an array of steel rollers (900 x 32 mm) driven at 120 rpm. The grinding action is produced by the tumbling of the sample and steel bars. The



He/O<sub>2</sub> flowrate 60 ml min<sup>-1</sup>: Combustion tube—quartz 430 x 20 mm, packing, Cr<sub>2</sub>O<sub>3</sub>/CuO/Ag, 1020° C: Reduction tube—quartz 300 x 20 mm, packing Cu, 600° C: Water trap—Mg(ClO<sub>4</sub>)<sub>2</sub>: GC column—400 x 6 mm, packing Carbosieve 60-80 mesh, 75° C: CO<sub>2</sub> trap — dipping autosampler, 7 X 100 mm tubes containing 7 ml CO<sub>2</sub> trapping scintillation cocktail.

Fig 1. Flow diagram of ANCA-MS system modified for CO<sub>2</sub> measurement and <sup>14</sup>CO<sub>2</sub> trapping.

mill enables many samples to be ground simultaneously and avoids moisture adsorption and cross contamination of samples. Fine grinding is used to increase sample homogeneity, it is not necessary for sample combustion. It is possible, for example, to analyse small intact legume root nodules.

The ground samples were weighed into tared tin cups (10 x 4 mm, Conroy Scientific, West Roxbury, Ma, USA). The tin cups were crimped and compressed into compact cylinders to pass through the autosampler of the C-N analyser (Roboprep, Europa Scientific, Crewe, England). Sample weights were adjusted to contain approximately 100 µg N (2-8 mg plant material, 60 mg soil).

**SAMPLE COMBUSTION:** The combustion tube (1020° C) contained  $\text{Cr}_2\text{O}_3$  granules as an oxidation catalyst (6), chopped CuO wire for oxidation of  $\text{CH}_4$  and chopped Ag wire to trap sulphur and halogens. The inner wall of the quartz tube in the combustion region was protected by a cylinder of nickel foil (40 x 16 x 0.125 mm).

The volume of  $\text{O}_2$  required for combustion of plant samples was estimated as equivalent to that for cellulose (approx.  $0.8 \text{ ml O}_2 \text{ mg}^{-1} \text{ dw}$ ) and that of the tin cup (22 mg) as 4 ml. The  $\text{O}_2$  pulse was set to ensure an excess in samples with the highest expected  $\text{O}_2$  consumption. For corn grain samples (8 mg) this was 15 ml, which gave approximately 4 ml of excess  $\text{O}_2$ . The  $\text{O}_2$  pulse for soil samples was set to 15 ml since the demand cannot easily be predicted due to varying organic matter content and unknown  $\text{O}_2$  requirement for the oxidation of the mineral component of the soil.

The timing of the  $\text{O}_2$  pulse and sample introduction was arranged such that immediate flash combustion of the sample was obtained. In our instrument this required a delay of 25 secs between the start of the  $\text{O}_2$  pulse and the introduction of the sample. This allowed the  $\text{O}_2$  to reach the upper surface of the combustion catalyst concurrent with the arrival of the sample. It has been argued that retention of N in the reduction stage of the C-N analyser is due to the formation of  $\text{NO}_x$  under the highly oxidizing conditions of the flash combustion and that  $\text{NO}_x$  formation could be minimized by allowing the sample to decompose in the less strongly oxidizing environment ahead of the  $\text{O}_2$  pulse (7). This was tested by removing the reduction stage, allowing NO to pass to the mass spectrometer, where it was measured as  $M/e$  30 with a retention time of 180 secs. Nitric oxide has been found to be the predominant nitrogen oxide formed during sample combustion (Preston, personal communication). The production of NO from soybean leaf samples remained constant at about 4% of total N when the sample introduction was advanced by 10 secs. This indicates that there is no advantage in premature introduction of the sample.

It is important to ensure that the system is leak free and that ash build up in the combustion tube is avoided. Ash was periodically removed using a length of copper tubing (6 mm id) attached to a vacuum line. The frequency of cleaning

depended on the nature of the samples, for soils the combustion tube was cleaned at least every 50 samples.

**REFERENCE SAMPLES:** Weighed samples of glycine ( $500\mu\text{g}$ ) were used as references for total N, total C and atom%  $^{15}\text{N}$ . These were used in preference to standards prepared from pipetted solutions because the reproducibility of micro-litre pipettes was inadequate for accurate elemental analysis. Standards were inserted in duplicate at the beginning of the run and after each group of 8 samples thereafter.

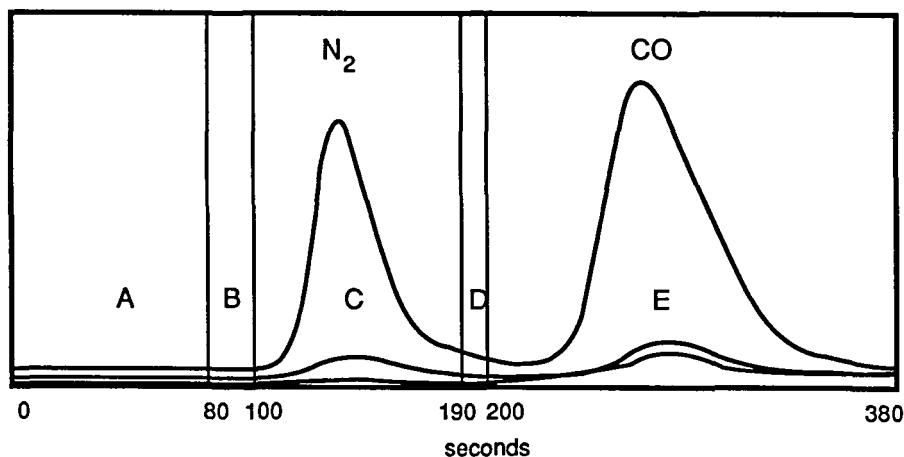
### TOTAL N AND $^{15}\text{N}$

**Mass Spectrometry:** The triple collector MS (Tracermass, Europa Scientific) was adjusted to center the M/e 29 beam in the middle collector. The splitter valve was opened to admit He carrier gas to an analyser pressure of  $5 \times 10^{-6}$  mbar. At this bleed rate the total signal for  $100\mu\text{g}$  N was  $2 \times 10^{-8}$  amp secs. Cycle timing was set as shown in Fig 2. , overall cycle time was increased from 300 to 380 secs over that required for N analysis alone due to the presence of CO (M/e 28) which is formed by fragmentation when  $\text{CO}_2$  is admitted to the ion source. This does not interfere with integration of the  $\text{N}_2$  peaks due to the separation of  $\text{N}_2$  and  $\text{CO}_2$  in the chromatography column.

Blank offsets were determined as the M/e 28, 29 and 30 signals resulting from an  $\text{O}_2$  pulse without sample introduction. This compensated for the  $\text{N}_2$  content of the  $\text{O}_2$  which is significant even in the best commercial grades. The N content of empty tin cups was not measurable and was ignored. Total N was calculated by the instrument software from the sum of the integrated M/e 28, 29 and 30 signals minus the blank offsets. Atom %  $^{15}\text{N}$  was calculated from:

$$\text{Atom\% } ^{15}\text{N} = 100 * \left[ \frac{\left( \frac{\text{M/e } 29}{\text{M/e } 28} \right)}{2 + \left( \frac{\text{M/e } 29}{\text{M/e } 28} \right)} \right]$$

The software also performed drift correction for total N and atom %  $^{15}\text{N}$  between each set of references. The M/e 30 signal was not used in the calculation of atom% for samples containing less than 5%  $^{15}\text{N}$ .



A = initial delay, B = first baseline correction period, C =  $N_2$  integration window, D = second baseline correction period, E = runoff period for  $CO$  signal.

FIGURE 2. Chromatogram of  $M/e$  28, 29 and 30 species in ANCA-MS instrument modified for  $CO_2$  and  $^{14}CO_2$  measurements.

**Kjeldahl Digestion:** Total N determinations on the plant and soil samples were made by Kjeldahl digestion using an aluminum block digester (Tecator) for comparison with total N values obtained by the ANCA-MS method. Aliquots of 100 mg plant sample or 1 g soil were digested for 5h with 3 ml  $H_2SO_4$  and a catalyst tablet (Kjeltab, Fisher Scientific) containing  $K_2SO_4$  (1.5 g) and Se (7.5 mg) in 100 ml graduated tubes. Ammonium was determined on a flow injection autoanalyser by a salicylate - nitroprusside method (Lachat Quickchem method # 10-107-06-2-E, Lachat Instruments, Mequon, WI, USA).

**Total C and  $^{14}C$  Determination:** Total C was measured as  $CO_2$  in the bypass gas stream from the mass spectrometer using a thermal conductivity detector (TCD) (Carle, Fullerton, CA, USA). The signal from the TCD was measured by a peak integrator (SpectraPhysics, San Jose, CA, USA). The integrator also controlled the operation of an autosampler (Isco, Lincoln, NE, USA) which was used to bubble the effluent gas stream from the TCD into tubes (13 x 100 mm) containing

7 ml of  $\text{CO}_2$  trapping scintillation cocktail (Harvey Instrument Co. Hillsdale, N.J, USA). As each sample was combusted, a signal from the C-N analyser initiated the integrator program. This in turn controlled the autosampler so that when the  $\text{CO}_2$  peak was expected, a stainless steel dipper tube (0.5 mm id) was lowered into the trapping cocktail. At the end of each sample cycle the dipper tube was raised and the tube cassette advanced to present a fresh  $\text{CO}_2$  trap for the next sample. After the sample run the scintillation cocktail was transferred to 28 mm scintillation vials and the collection tubes rinsed with further 7 ml aliquots of scintillation cocktail. The radioactivities of the samples were determined using a scintillation counter (Beckman Instruments, Fullerton, CA, USA), quench correction was by the H-number method using external quench standards.

Recovery of  $^{14}\text{C}$  was tested using  $^{14}\text{C}$ -glucose and  $^{14}\text{CO}_2$ -labelled soybean leaves. Aliquots (50, 20 and 5  $\mu\text{l}$ ) of a  $^{14}\text{C}$ -glucose solution containing 26 Bq  $\mu\text{l}^{-1}$  were added directly to the  $\text{CO}_2$ -trapping scintillation cocktail. A parallel series were adsorbed on cellulose filter paper (3 x 3 mm) contained in tin sample cups, dried at 60° C and combusted in the ANCA-MS system. The soybean leaf samples were either combusted as above or were solubilized in methylbenzethonium hydroxide (hyamine hydroxide) following the method of Fuchs and deVries (4).

## RESULTS AND DISCUSSION

**TOTAL N AND  $^{15}\text{N}$ :** Analyses were conducted to compare the accuracy of total N and  $^{15}\text{N}$  measurements when the instrument was set for both N and C determination to that obtained when only N was admitted to the analyser and to compare these to total N values obtained by the Kjeldahl method. Total N determined by the ANCA-MS method differed significantly from the values obtained by Kjeldahl digestion in two of the seven sample materials (Table 1). The Kjeldahl method, which was not modified to include nitrate, failed to recover about 7% of the N contained in tomato leaf tissue (NBS std 1573, nominally 5.0% N). It is possible that this material contained a high concentration of  $\text{NO}_3^-$ . Both methods gave slightly lower total N values for citrus leaf (NBS std 1572) than the quoted but uncertified value of 2.86%N, though for the Kjeldahl analyses the difference was not significant. We have no explanation for the lower total N values obtained

TABLE 1. Total N content of plant and soil samples determined by ANCA-MS and Kjeldahl analysis

sample	Total N(%) $\pm$ SD (n=8)		
	ANCA-MS	KJELDAHL	
Soybean leaf	5.129 $\pm$ 0.051	5.179 $\pm$ 0.132	n.s.
Citrus leaf	2.701 $\pm$ 0.035	2.777 $\pm$ 0.160	n.s.
Tomato leaf	5.013 $\pm$ 0.038	4.635 $\pm$ 0.243	***
Corn grain	1.556 $\pm$ 0.032	1.524 $\pm$ 0.073	n.s.
Soil 1	0.173 $\pm$ 0.002	0.188 $\pm$ 0.006	***
2	0.153 $\pm$ 0.002	0.148 $\pm$ 0.010	n.s.
3	0.100 $\pm$ 0.003	0.093 $\pm$ 0.008	n.s.

n.s. - not significant, \*\*\* -  $p < 0.001$  (Unpaired, two-tailed t-test)

by the ANCA-MS method for citrus leaf and soil 1. It is notable that the standard deviations of the mean total N values for the ANCA-MS method were considerably lower (50 - 30%) than for the Kjeldahl. Much of the variation in the Kjeldahl values can be attributed to the variability of the flow injection autoanalyser method for ammonium determination which typically showed a coefficient of variation of about 2% for repeated standard samples.

The possible interference of CO with the measurement of atom%  $^{15}\text{N}$  was tested. Samples were analysed both with the instrument set for normal  $^{15}\text{N}$  analysis, where the gas stream was scrubbed of  $\text{CO}_2$  before admission to the mass spectrometer, and in the modified mode where  $\text{CO}_2$  was allowed to enter the analyser. The atom%  $^{15}\text{N}$  values for the test samples (Table 2) show that the presence of  $\text{CO}_2$  in the gas stream need not interfere with the analysis of  $\text{N}_2$ . Reproducibility was comparable to that found when the instrument was set solely for  $^{15}\text{N}$  analysis. It is important however, that the retention times for  $\text{N}_2$  and  $\text{CO}_2$  remain reasonably stable. Any large variation in retention time can displace the  $\text{N}_2$  peak from the center of its integration window (region C, Fig. 2) resulting



either in low total N values, due to increased baseline correction, or erroneous atom% measurements resulting from inclusion of signal due to CO in the integrated values for  $\text{N}_2$ . Slight fluctuations in flow rate are inevitable because the consumption of  $\text{O}_2$  and production of  $\text{N}_2$  and  $\text{CO}_2$  during sample combustion vary with sample size and composition. Larger fluctuations can be caused by increased resistance to flow due, for example, to solidification of the  $\text{Mg}(\text{ClO}_4)_2$  in the water trap. This has the effect of increasing the backflush of carrier gas through the autosampler.

There is typically carryover or "memory" between samples such that when consecutive samples of widely different atom %  $^{15}\text{N}$  content are analysed, the second result is influenced by the former. Kirsten and Hesselius (8) showed that memory is due to retention of complexed  $\text{NO}_x$  on the copper reduction stage. This is illustrated in Table 3 where a series of 15 analyses are listed. The series includes transitions from soybean leaf material at natural abundance to moderately enriched corn grain and back to soybean leaf. After the transition from background  $^{15}\text{N}$  levels to 1.25 atom %  $^{15}\text{N}$ , the first two analyses of corn grain show atom %  $^{15}\text{N}$  values which are significantly depressed compared to the "true" values.

The data from the first transition (0.366 to 1.251) suggest that about 5  $\mu\text{g}$  of the sample N may be retained and subsequently released to contaminate later samples. In the second transition from corn at 1.251 atom% to soybean leaf at 0.366 atom%, the memory effect is much smaller (0.35 $\mu\text{g}$  N). This difference could be explained if the combustion of soybean leaf tissue resulted in the formation of more  $\text{NO}_x$  than did the combustion of the corn grain sample. This is likely because the C/N ratio of the soybean leaf tissue (8:1) was much lower than that of the corn grain (27:1) thus smaller samples (2 mg) were combusted with an  $\text{O}_2$  pulse tailored to the larger corn grain samples (8 mg). This resulted in an excess of  $\text{O}_2$  for combustion of the soybean leaf samples which probably increased the formation of  $\text{NO}_x$ .

The sample memory effects can be minimized by careful attention to the combustion conditions and the size of the  $\text{O}_2$  pulse but cannot yet be eliminated. Our current strategy is to limit the problem by careful organization of sample runs to minimize transitions between samples of widely

TABLE 2. Atom%  $^{15}\text{N}$  content of plant and soil samples measured by ANCA-MS with and without  $\text{CO}_2$  trapping .

Sample	N-only mode		N + $\text{CO}_2$ mode	
	Atom%	$\pm\text{SD}$	Atom%	$\pm\text{SD}$ (n = 8)
Soybean leaf	0.3676	0.0002	0.3672	0.0004
citrus leaf	0.3672	0.0002	0.3678	0.0003
tomato leaf	0.3667	0.0003	0.3666	0.0005
corn grain	0.3706	0.0004	0.3710	0.0002
Soil 1	0.3696	0.0013	0.3698	0.0005
2	0.3722	0.0006	0.3725	0.0010
3	0.3707	0.0012	0.3703	0.0008

TABLE 3. The effect of sample memory on the determination of  $^{15}\text{N}$ .

Series	1		2		3	
Sample	Soybean leaf		Corn grain		Soybean leaf	
	atom %	atom%	carryover*		atom %	carryover
			(% N)			(% N)
1	0.36654	1.22531	2.98		0.36877	0.25
2	0.36635	1.23754	1.60		0.36707	0.05
3	0.36643	1.25148	0.02		0.36684	0.03
4	0.36665	1.25199			0.36671	
5	0.36650	1.25160			0.36642	

\*carryover or sample memory calculated from:

$$\%N_{\text{mem}} = 100 - \left[ \frac{(A_m - A_p)}{(A_t - A_p)} \times 100 \right]$$

where:

$N_{\text{mem}}$  = N from previous sample series,  $A_m$  = measured atom%  $^{15}\text{N}$ ,  $A_p$  = atom%  $^{15}\text{N}$  in previous sample series,  $A_t$  = 'true' atom %  $^{15}\text{N}$ .

differing composition, and where these are unavoidable, to buffer the transition with one or more dummy samples.

**TOTAL C AND  $^{14}\text{C}$ :** The trapping of  $^{14}\text{CO}_2$  produced by combustion in the ANCA-MS instrument was essentially complete (Table 4). The admission of sample gas to the mass spectrometer did not significantly deplete the gas flow from the combustion unit. Although the solubilization of soybean leaf tissue in methylbenzethonium hydroxide appeared complete, the counts obtained were 5% lower than those obtained from the ANCA-MS instrument.

It is possible to obtain cassettes for the autosampler which hold normal scintillation vials and these could be used directly for  $\text{CO}_2$  trapping. However, the path length for bubbles in the trapping cocktail would be shortened and this might reduce trapping efficiency. Sample cross contamination of about 0.5% was caused by transfer of scintillation cocktail by the dipper tube. This could be avoided by moving the dipper tube to a wash position between samples, but would require the continuous disposal of contaminated washing solvent.

### CONCLUSIONS

The combination of ANCA-MS for analysis of total N and  $^{15}\text{N}$  with collection and measurement of  $^{14}\text{CO}_2$  enables the rapid and convenient elemental and isotopic analysis of plant and soil samples from dual tracer experiments. Precision was at least equivalent to that of conventional separate analyses for total N, total C and  $^{14}\text{C}$ . The external precision for  $^{15}\text{N}$ , of about  $\pm 1\text{‰}$ , was adequate for most tracer work. Studies exploiting natural isotopic fractionation and those where pool dilution is very great, may still require a dual inlet mass spectrometer. The small samples required can be advantageous where it is difficult to obtain sufficient material for conventional analysis. However, the greatest advantage of the method is the speed and ease with which samples from tracer experiments can be analysed. We were able, for example, to measure  $^{14}\text{CO}_2$  uptake and  $^{15}\text{N}_2$  incorporation in soybean root nodules and to complete the procedure from exposure to isotopic analysis of the material within one day. This allows the rapid development and testing of hypotheses without the weeks or months of delay usual in  $^{15}\text{N}$  work.

TABLE 4. Recovery of  $^{14}\text{C}$  from soybean leaves and  $^{14}\text{C}$ -glucose-impregnated filter papers.

Sample	%C	$\pm\text{SD}(n=8)$	Radioactivity (Bq $\pm$ SD)			
			ANCA-MS		Comparison	
Soybean leaf	44.10	$\pm 0.38$	2083	$\pm 28$	Solubilized 1931 $\pm 89$	
Filter paper+ $^{14}\text{C}$ -glucose					Direct addition	
50 $\mu\text{l}$	43.24	$\pm 0.96$	1405	$\pm 7$	1405	$\pm 19$
20 $\mu\text{l}$	43.31	$\pm 0.78$	561	$\pm 8$	585	$\pm 11$
5 $\mu\text{l}$	43.51	$\pm 0.81$	127	$\pm 17$	131	$\pm 15$

Sample memory in the C-N analyser may limit the accuracy of  $^{15}\text{N}$  analyses unless care is taken in the organization of sample batches and in the control of combustion conditions. The greatest potential for improvement of the ANCA-MS method is in the optimization of the sample combustion and reduction stages which could both decrease memory problems and increase the flexibility of sample batch organization.

The  $^{14}\text{CO}_2$  collection method, though not truly "on-line", has advantages over possible continuous flow systems using solid state or gas proportional detectors. Trapping is complete, counting efficiency is high and counting time and therefore resolution is not limited by the residence time of the  $^{14}\text{CO}_2$  peak in a flow detector.

Our unit has analysed approximately 8000  $^{15}\text{N}$  and 1000  $^{15}\text{N}$  -  $^{14}\text{C}$  samples in 18 months of operation, when fully utilized, 500 samples per week can be processed. Most of these samples were analysed by graduate students with supervision by the primary operator. Downtime for maintenance and repair has totalled about 6 weeks due mainly to two breakdowns which occurred shortly after installation and required the return of electronic modules to the manufacturer.

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