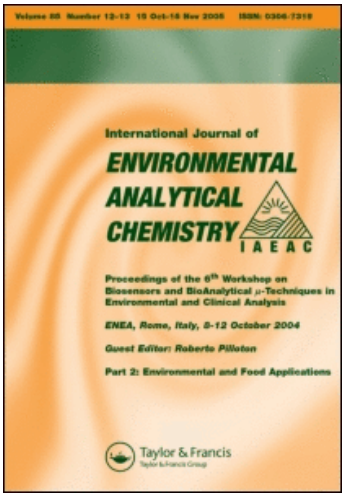

International Journal of Environmental Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713640455

Microdetermination of Carboxyl Groups in Fulvic Acid and Related Polycarboxylates

Joseph Evans[a]; Phillip W. Albro[a]
[a] Laboratory of Molecular Biophysics, National Institute of Environmental Health Sciences, Research Triangle Park, NC

To cite this Article Evans, Joseph and Albro, Phillip W.(1986) 'Microdetermination of Carboxyl Groups in Fulvic Acid and Related Polycarboxylates', International Journal of Environmental Analytical Chemistry, 24: 2, 133 — 141
To link to this Article: DOI: 10.1080/03067318608076464
URL: http://dx.doi.org/10.1080/03067318608076464

Intern. J. Environ. Anal. Chem., 1986, Vol. 24, pp. 133–141
0306-7319/86/2402-0133 $18.50/0

Printed in Great Britain

Microdetermination of Carboxyl Groups in Fulvic Acid and Related Polycarboxylates

JOSEPH EVANS and PHILLIP W. ALBROt

Laboratory of Molecular Biophysics, National Institute of Environmental Health Sciences, PO Box 12233, Research Triangle Park, NC 27709

(*Received September 11, 1985*)

A radiochemical procedure was used to quantify carboxyl content of fulvic acid. Tritium from tritiated water exchanged with carboxyl protons in fulvic acid and were then locked into the fulvic acid structure by diazomethane methylation. Liquid scintillation counting yielded quantifiable results using 100 microgram quantities of fulvic acid. Values obtained were comparable within 2% to those obtained with a $Ca(OAc)_2$ titration procedure for carboxyl determination requiring 50 milligrams of polycarboxylate.

KEY WORDS. Fulvic acid, carboxyl groups, radioassay, quantification.

INTRODUCTION

Humic and fulvic acids are defined as those organic components of natural waters that are not extractable into organic solvents; the former precipitates at pH 1 while the latter does not.[1] During studies of the binding properties of fulvic acid from different sources we found it necessary to compare small batches as to carboxyl group content. Standard methods for this determination exist,[2,3] but require relatively large amounts of material (on the order of 50 mg, ref. 3). As the purification of even mg amounts of fulvic acid is time consuming and tedious,[1] we needed a method for carboxyl group determination applicable to submilligram quantities of polycar-

†Author to whom correspondence should be addressed.

boxylates. Since analyses for phenolic hydroxyl groups[6] had indicated that our preparations of fulvic acid were extremely low in this functionality, we decided to take advantage of the characteristics of the diazomethane esterification reaction as a means of radiochemical determination of carboxyl groups.

Diazomethane has been shown to introduce $-CH_2-$ groups into cyclic structures of humic or fulvic acid.[4] For this reason we could not use radiolabeled diazomethane for carboxyl group determination. Preliminary experiments involving proton nuclear magnetic resonance spectroscopy in D_2O revealed that essentially all of the protons in fulvic acid that were capable of exchanging did so very rapidly, exchange being complete in less than 90 seconds. This being the case, radioactivity could be introduced and "fixed" as described below.

MATERIALS AND METHODS

Fulvic acid used in our experiments was collected from Singletary Lake and Black Lake, NC. It was isolated by the humics group at the University of North Carolina, Chapel Hill. The isolation procedure used was as described by Thurman and Malcolm.[1]

Exchange; esterification; re-exchange

Start with duplicate samples containing 100 μg of fulvic acid in 100 μl of deionized water aliquoted into 0.5 dram glass vials. Benzene pentacarboxycylic acid (Pflatz and Bauer; Stanford, CT) was used as a separate reference standard in each set of samples to correct for variations in dilution of ^3H_2O by moisture in the diethyl ether as well as humidity in the air. The samples and standards are placed in a vacuum desiccator over Drierite ($CaSO_4$, Hammond Co.; Xenia, OH) and NaOH pellets (Allied Chemical; Morristown, NJ) until dry (usually 24 hours).

To each of the dry samples and standards add 20 μl of ^3H_2O (activity used 2.49×10^8 DPM/g, obtained from New England Nuclear, Boston, MA) and vortex. Allow 2 minutes to assure complete exchange of tritium with hydrogen in the samples and then add 1 ml of diazomethane in ether previously generated as described

by Fales *et al.*[5] using N-methyl-N-nitro-N-nitrosoguanidine (Aldrich; Milwaukee, WI). The vials are securely capped with a teflon-lined screw cap, and placed on a shaker for 1 hour.

Pure paraffin (Gulfwax), 1 mg, is then added to the vials containing benzene pentacarboxylic acid to prevent formation of a dry dust. The samples and standards are dried at 38°C under a stream of N_2 for 10–20 minutes. To the now methylated samples one drop of non-labeled, deionized water is added, the vials vortexed, and standards as well as samples are put back in the vacuum desiccator overnight. This step is repeated the following day.

Radioassay procedure

To the dried samples and standards were added a few drops of methanol or acetonitrile–methanol (1:1, v/v) to dissolve the solid residue. Each small vial was then crushed, using a pair of pliers, inside a larger scintillation vial. To these were added 15 ml of Riaflour (New England Nuclear; Boston, MA) and the incorporated (non-exchangeable) tritium radioassayed using a liquid scintillation counter (Packard, Tri Carb 4530).

Calculation of results

1) The benzene pentacarboxycylic acid is used to determine the *effective specific activity* of the tritiated water by the following method:

$$\textit{effective specific activity} \equiv \frac{\text{equiv. } COOCH_2{}^3H \text{ counted}}{\text{equiv. COOH originally present}}$$

$$\text{equiv. } COOCH_2{}^3H \equiv \frac{\text{DPM observed (benzene pentacarboxycylic acid)}}{\text{DPM/g (Activity of } {}^3H_2O \text{ used)} \times 18\,\text{g/equiv. } {}^3H_2O \times 1/2^*}.$$

*The specific specific activity per ^3H is 1/2 that of ^3H_2O hence the 1/2 used above.

equiv. COOH originally present

$$\equiv \frac{\text{gm. benzene pentacarboxcylic acid}}{298} \times 5.$$

*5 equiv. COOH per mol benzene pentacarboxcylic acid.

**298 = F.W. of benzene pentacarboxcylic acid.

2) Equivalents of carboxyl in the fulvic acid samples is computed as follows:

$$\frac{\text{equiv. } COOCH_2{}^3H \text{ (counted in methylated fulvic acid)}}{\text{effective specific activity}}$$

equiv. $COOCH_2{}^3H$
(counted in methylated fulvic acid)

$$\equiv \frac{\text{DPM observed (methylated fulvic acid)}}{\text{DPM/g (Activity of } {}^3H_2O \text{ used)} \times 18 \text{ g/ea. } {}^3H_2O \times 1/2}.$$

3) Equivalents of carboxyl per gram of fulvic acid is then computed.

$$\frac{\text{equiv. COOH in sample}}{\text{gm. of fulvic acid}}.$$

3a) To obtain a weight percent of carboxyl in fulvic acid

weight percent $\equiv$ (equiv. COOH/gm. of fulvic acid) $\times$ 45 $\times$ 100.

*45 = F.W. of COOH.

Control experiments

To test the exchange of carboxyl protons with water we put a few milligrams of benzene pentacarboxcylic acid into two separate NMR tubes and to one added d_6-DMSO (Merck, Sharp & Dohme: Kirkland, Quebec, Canada) and to the other 100% D_2O (Aldrich; Milwaukee, WI). Noting the time when we dissolved our standard in D_2O would allow us to determine how much time is required for a complete exchange of hydrogen with deuterium by comparing the NMR spectra of both solutions. The NMR spectrum of benzene

pentacarboxcylic acid in d_6-DMSO would show no exchange with deuterium while the D_2O NMR spectrum examined at several different time points would show exchange with respect to time. Used for all NMR spectra was a Nicolet QE 300, 300 MHz NMR spectrophotometer, 2.05 seconds/scan.

As indicated previously it is necessary to run a benzene pentacarboxcylic acid standard with each set of samples to determine an effective specific activity. To better understand why this varies from run to run we ran an experiment using benzene pentacarboxcylic acid. We exchanged the protons with D_2O instead of ^3H_2O, methylated our standard, dried it, and dissolved it in d_6-DMSO. By comparing the NMR spectrum of this sample with a benzene pentacarboxcylic acid sample that was methylated but not exchanged with D_2O we could determine what percentage of our methyl groups were $—CH_2D$ and if any $—CH_3$ groups still remained.

To evaluate the percentage esterification by our diazomethane methylation procedure we methylated, as previously described, benzene pentacarboxcylic acid samples and fulvic acid samples. These were dried, pressed into pellets with KBr (Perkin Elmer; Norwalk, CT) and infrared spectra were run on a Perkin Elmer 1320 infrared spectrophotometer. These IR spectra were compared to those of non-methylated benzene pentacarboxcylic acid and fulvic acid samples.

Fulvic acid contains exchangeable protons other than carboxyl. This is why the re-exchange of tritium with hydrogen after methylation is necessary. To test for complete re-exchange of protons with groups other than carboxyl we used an hippuric acid (Figure 2) standard which has an exchangeable proton attached to a nitrogen, not subject to methylation under these conditions, as well as having an exchangeable carboxyl proton. It was exchanged, methylated and re-exchanged as described for fulvic acid and radioassayed with a standard benzene pentacarboxcylic acid sample for comparison.

Finally to compare our method of carboxyl determination with a more standard method we chose to run one sample of fulvic acid both by the present method and by the procedure described by Schnitzer and Gupta.[2]

RESULTS

The results of carboxyl determination by the tritium exchange

procedure are shown for different samples of fulvic acid in Table I. The comparison of the results obtained for one sample of fulvic acid by the tritium exchange method versus the $Ca(OAc)_2$ method[2] is shown in Table II. As shown in the table the two separate methods gave similar results confirming the validity of our tritium exchange procedure. The $Ca(OAc)_2$ method required 50 mg of fulvic acid. The different samples of fulvic acid in Table I were collected separately at various times during the year and show some batch to batch variation.

When we examined the proton NMR spectra of the two samples of benzene pentacarboxcylic acid dissolved in d_6-DMSO and D_2O respectively, our sample in DMSO showed two peaks as expected.

TABLE I

Comparison of carbonyl content of various samples of fulvic acid

Sample[a]	1	2	3	4
meq. COOH / gm. fulvic acid	5.78 ±0.46	3.65 ±0.16	4.00 ±0.25	3.65 ±0.11
Weight %	26.0±2.1	15.4±0.7	18.0±1.1	16.4±0.5

[a]Samples 1, 2 and 4 from Lake Singletary, sample 3 from Black Lake.

TABLE II

Comparison, by two independent methods of carboxyl content of one fulvic acid sample

Fulvic acid sample 1		
Method	Tritium exchange	$Ca(OAc)_2$ (4)
meq. COOH / gm. fulvic acid	5.78±0.46	5.19±0.13
Weight %	26.0±2.1	23.4±0.6

The singlet at 8.6 ppm (tetramethyl silane reference), reflected the single hydrogen on the benzene ring, while a rather broad peak at 13.7 ppm represented the collection of the 5 hydrogens on the carboxyl groups. The sample in D_2O showed the peak at 8.6 ppm but no peak at 13.7 ppm after only $1\frac{1}{2}$ minutes, indicating complete exchange of hydrogen by deuterium on all 5 carboxyls. This confirmed the exchange was both rapid and complete.

The NMR spectrum of the benzene pentacarboxcylic acid exchanged with D_2O and then methylated, compared with the spectrum of methylated, non-exchanged benzene pentacarboxcylic acid clearly demonstrated the need to determine the "effective" specific activity of the tritiated water for each batch of samples processed. We knew from our previous results that the exchange is essentially 100%; however as shown in Figure 1, since there is a slightly different shift for a $—CH_2D$ group from a $—CH_3$ group, 78% of our exchanged standard was $—CH_2D$ while the remaining fraction was $—CH_3$. This indicated re-exchange with hydrogen had taken place before methylation. Evidently there is a variable dilution effect from run to

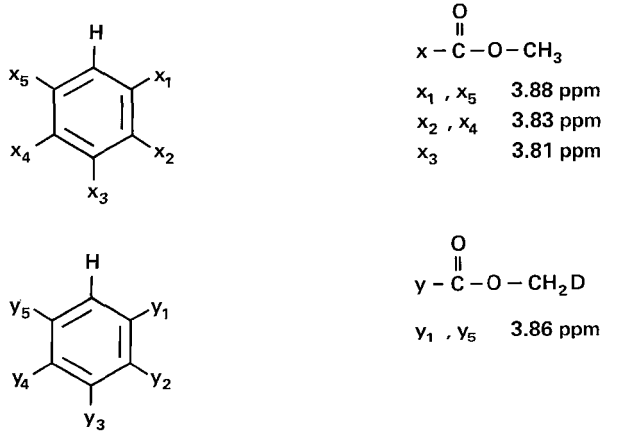

FIGURE 1 Proton NMR spectroscopy of native and D_2O-exchanged benzene pentacarboxylate. The peak area ratios indicated a 78% trapping of ^2H in the methyl groups for this sample.

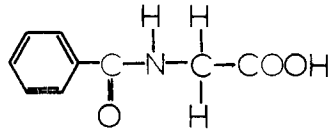

FIGURE 2 Structure of hippuric acid.

run probably dependent upon the humidity in the lab air or trace amounts of water in the ether. This is why it is necessary to include a standard of known COOH composition with each sample run.

Complete esterification of our fulvic acid and benzene pentacarboxcylic acid was verified by infrared spectroscopy. (Not shown. Copies of the NMR and IR spectra are available from the authors on request.) The absence of any peak from 2,650 to 2,000 cm^{-1} (COOH region) in our methylated samples, as was previously present in our non-methylated samples, and the emergence of a large sharp peak at 1,730 cm^{-1} (methyl ester carbonyl) in our methylated sample confirmed complete esterification in both the fulvic acid and benzene pentacarboxcylic acid (within the limits of detection of infrared spectroscopy, roughly 3% —COOH detectable).

Finally, our control experiment using hippuric acid to test for the complete re-exchange of tritium, other than carboxyl, gave results that correlated precisely with those recorded for benzene pentacarboxcylic acid. The effective fixation of ^3H in the methylated hippuric acid was $85.7\% \pm 1.7\%$ and for benzene pentacarboxcylic acid $84.2\% \pm 2.5\%$. This confirmed the re-exchange procedure was complete.

DISCUSSION

In performing this assay it is important to use quantities similar to those stated. We found new problems occurred when we scaled down or up from the presented quantities. For example, 100% methylation of the fulvic acid is achievable only when the diazomethane is available in excess, possibly because of competing side reactions. Incomplete methylation was easily observable in infrared spectra of samples containing too little diazomethane. The same problem can occur if too much tritiated water is used, causing two

phases, ether and water. When this happens the fulvic acid remains in the water phase and the diazomethane in the ether phase, hence incomplete methylation. Reaction time and continuous shaking are also important to assure 100% methylation.

While this method is designed for fulvic or humic acid some researchers may find it useful for other polyanions as well. It was designed to be used when only small amounts of a high molecular weight organic material are available.

References

1. E. M. Thurman and R. L. Malcolm, *Environ. Sci. Technol.* **15,** 463 (1981).
2. M. Schnitzer and V. C. Gupta, *Soil. Sci. Soc. Amer. Proc.* **39,** 274 (1965).
3. L. Blom, L. Eddhauser and D. W. van Kreuelen, *Feul* **36,** 135 (1957).
4. M. Schnitzer, *Soil Sci.* **117,** 94 (1974).
5. H. M. Fales, T. M. Jaouni and J. F. Babashak, *Anal. Chem.* **45,** 2302 (1973).
6. J. Chrastil, *Anal. Chem.* **47,** 2293 (1975).