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OXIDATION–LIQUID SCINTILLATION RADIOASSAY OF PHENOLICS FROM CELLULOSE THIN-LAYER CHROMATOGRAPHY PLATES

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SUMMARY

A new technique is described for the radioassay of ^{14}C labeled phenolics removed from thin-layer chromatographic plates using cellulose nitrate solution. Samples are transferred to liquid scintillation vials for direct oxidation and $^{14}CO_2$ absorption preparatory to counting. High-efficiency counting results are recorded for the low-activity-level phenols with and without selective chromogenic spray detection. The method includes greater economy, and superior sample preparation and handling characteristics as advantages over other methods currently in use.

INTRODUCTION

Cellulose thin-layer chromatography (TLC) definitively separates phenolic and phenolic glycoside mixtures extracted from plant tissues[1,2]. The inherent efficiency of the chromatography suggests its potential application in the separation and radioassay of phenolic and other organic metabolites in radioactive feeding experiments.

Anticipated low levels of incorporation of radioactive precursors into phenols in most feeding experiments effectively eliminate autoradiography[3] or chromatographic plate scanning[4] as means of component chromatographic location and radioassay, on the basis of excessive time or equipment requirements. Therefore, the evaluation of these low-level beta emitters requires a radioassay method of high efficiency for the labeled compounds specifically located and quantitatively removed from the chromatographic support.

Liquid scintillation radioassay is recognized as the best means of measuring radioactivity on TLC plates[5]. The scintillation measurements can be accomplished by either elution analysis (solubilization, suspension or emulsification) or oxidation–absorption analysis. High-efficiency elution analysis of labeled amino acids on paper chromatograms has been achieved using Bio Solv BBS-3 with water to dissolve the chromatographic support prior to counting[6]. This method, applied to cellulose supports, reduces or eliminates physical quenching problems experienced in the radioassay of samples on solid supports[7].

Phenolic compounds present additional limitations to high-efficiency measurement of radioactivity on chromatographic supports, including: limited solubility in many scintillation solvents, natural chemical luminescence or chemical quenching

(photon absorptivity), and chemical or physical quenching of phenol–chromogenic spray complexes.

Oxidation–absorption methods can eliminate the solubility, quenching and luminescence problems of phenols and perhaps of the phenol–chromogenic spray complexes while offering near quantitative measurement of their radioactivity as $^{14}CO_2$ and $^{3}H_2O$ (refs. 8, 9). The oxidation–absorption method has recently been adapted to commercial instrumentation for the automatic oxidation of biological samples for liquid scintillation analysis of ^{14}C and/or ^{3}H (ref. 10). While the commercial instruments are convenient and widely applicable, their usefulness in a particular research program is governed by equipment costs and initial sample preparation time.

A desirable economical alternative to the automatic instrumentation as particularly applied to samples from self-prepared cellulose TLC plates would include: (a) a quantitative method for removal of radioactive samples from the thin-layer plate and (b) an oxidation procedure producing liquid scintillation samples utilizing common laboratory materials. The sample preparation and oxidation technique described in this investigation meets these criteria.

METHODS AND MATERIALS

Preparation of chromatographic plates

Thin-layer cellulose plates were prepared by blending 20 g of microcrystalline cellulose (Avicel*, E.M.C. Corp., American Viscose Div., Marcus Hook, Pa., U.S.A.) with 80 ml of water in a Waring blendor for 15 sec. The resulting slurry was spread on five (8 × 8 in.) glass plates to a thickness of 0.25 mm using a Shandon apparatus. The plates were then air dried for at least 24 h before use.

Preparation of ^{14}C standard solution

A 200-μl aliquot of DL-[U-^{14}C]-phenylalanine (New England Nuclear Corp., Boston, Mass., U.S.A.) (10 μCi/ml) was diluted to 10 ml with distilled water to yield a stock solution of 556 dpm/μl. Portions of 5 μl (2780 dpm) of the ^{14}C standard solution were applied by microsyringe to all radioactive chromatographic samples.

Preparation of chromatographic sample removing solution

Solid cellulose nitrate (Parlodion®, Mallinkrodt, St. Louis, Mo., U.S.A.) was dissolved in 95% ethyl alcohol–diethyl ether (50:50 v/v) to achieve a 3% w/v solution. The solubilization may take several days to complete. Three ml (3% v/v) of black ink (Esterbrook Pen Co., Lewisburg, Tenn., U.S.A., Stock No. T-104) were added to the cellulose nitrate solution to aid in light absorption during oxidation. The cellulose nitrate solution was refrigerated when not in use.

Oxidation sample preparation

Using a plastic circle template, 10-mm-diameter circles were scribed through the dry cellulose layer to the glass plate with a dissecting needle. To each circle

* Reference to a company and/or product named by the Department is only for purpose of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

5 μl of the ^{14}C standard solution were applied (excepting blanks). Appropriate amounts of prepared solutions of pinosylvin (3,5-dihydroxystilbene), quercetin (3,3′,4′,5,7-pentahydroxyflavone) in methanol and D-phenylalanine in distilled water (henceforth designated as external standards) were added to predesignated chromatographic spots. After applying the ^{14}C standards and the external standards, the chromatograms were allowed to dry for 1 h. The samples were then uniformly sprayed with specific chromogenic spray reagents $FeCl_3$–$K_3[Fe(CN)_6]$ (3% aqueous, equal volumes), bis-diazotized benzidine[11], and ninhydrin. The first two spray reagents were allowed to dry and react for 2 h while the ninhydrin-sprayed samples were heated briefly and allowed to cool and react for 1 h. Five replicates of each sample were prepared for evaluation.

Using an eye dropper, the cellulose nitrate solution was carefully applied to the scribed oxidation samples such that one drop would cover the 10-mm spot to the scribed perimeter. The treated spot was allowed to air dry until the circle began to curl and detach itself from the glass plate (Fig. 1). The average weight of the detached circles was determined to be 6.5 mg.

Each detached circle was carefully placed in a wire stand fashioned from 24-gauge platinum–iridium (15%) wire (Fig. 1). The sample and stand were then placed in a borosilicate glass liquid scintillation vial so as not to touch the sides. The sample was then ready for oxidation.

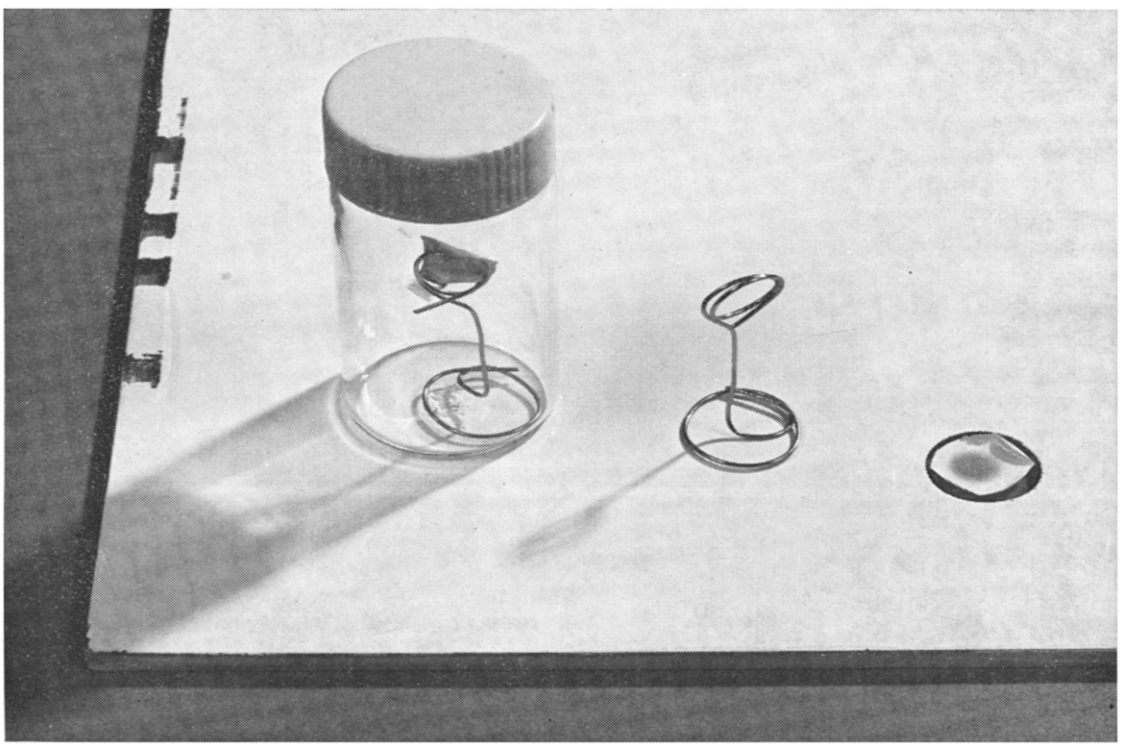

Fig. 1. Oxidation sample beginning to detach from thin-layer cellulose plate, oxidation stand and sample on stand preparatory to oxidation–absorption.

Sample oxidation

To vials containing oxidation samples, 1 ml of cold ethanolamine (50% v/v in methyl cellosolve) was pipetted to the bottom of the vial. A small amount of high-vacuum silicone grease was applied to the vial lip and the vial was flushed with a gentle stream of oxygen for 3–5 sec. The vial was quickly and tightly sealed and transferred to a fume hood for oxidation (a maximum of 5 vials could be prepared and combusted at any one time).

Oxidation was accomplished according to Gupta[9] using a focused infrared light source (Arthur H. Thomas Co. Model No. 6516-S10) to concentrate an external heat source on the sample inside the vial until ignition occurred. After complete ignition, the vial was swirled and immediately placed in the refrigerator. Fifteen min later the vial was removed and opened to add 13 ml of the scintillation cocktail (5.5 g PPO in 666 ml toluene plus 333 ml methyl cellosolve). The vial was then swirled and the wire stand removed. The vial was returned to the refrigerator until counted. Note: Ignitions should be carried out with proper shielding and hand protection for the worker. In the study, 3 vial explosions occurred in about 450 oxidations. These explosions were directly attributable to vial temperature and ethanolamine volatility just prior to oxidation.

Suspension sample preparation

Suspension sample scribed cellulose circles were scraped from the glass plate with a razor blade on to glassine paper. These samples were then poured into scintillation vials, prepared fluor (Liquifluor, New England Nuclear Corp.) was added, the vials swirled briskly and placed in a refrigerator preparatory to counting.

Standard blanks and activity ratios

Five replications of ^{14}C standards (excluding external phenol and phenylalanine standards) were prepared for each suspension and oxidation treatment. These standard blanks were averaged to an adjusted standard count rate for each treatment reflecting the degree of quenching or luminescence attributable to the mode of sample removal, chromogenic spray reagent, scintillation cocktail and method of ^{14}C analysis exclusive of the external standards. The adjusted ^{14}C standard count rates were then used to determine the activity ratio of each sample according to the formula:

$$\text{Activity Ratio (AR)} = \frac{\text{observed sample counts per min.}}{\text{adjusted standard counts per min.}}$$

The AR values are used to more directly compare counting results between treatments.

Sample liquid scintillation counting

Each scintillation sample was counted for three 5-min periods on a Packard Tri-Carb Liquid Scintillation Counter (Model No. 1200). The counter incorporated an automatic external standard (AES) for counting efficiency evaluation. The recorded AES values were corrected to a calibrated curve of real efficiencies determined in a separate operation on the instrument[12].

Statistical analysis of sample treatment

The relative precision of the two ^{14}C measuring techniques described in this investigation was evaluated in a one-way classification analysis of variance[13] of the observed count rates for each sample treatment over all concentrations of externally applied standard. The significance of lack of precision for each sample treatment is evaluated by comparing a calculated F value (the ratio of the between to the within count-rate mean squares) to a tabled F value based on the degrees of freedom between and within at the 5% probability level.

TABLE I

SCINTILLATION ACTIVITY RATIOS, SUSPENDED SAMPLES

NS = No spray; Fe = $FeCl_3$–$K_3[Fe(CN)_6]$; BDB = bis-diazotized benzidine; Nin = ninhydrin.

Sample	*Chromogenic treatment*			
	NS	*Fe*	*BDB*	*Nin*
^{14}C Standard phenylalanine (2780 dpm)	1.00	1.00	0.81	1.12
^{14}C Stand. + stilbene (2 μg)	1.18	—	—	—
^{14}C Stand. + stilbene (5 μg)	1.24	—	—	—
^{14}C Stand. + stilbene (10 μg)	1.22	—	—	—
^{14}C Stand. + quercetin (5 μg)	0.99	0.91	0.88	—
^{14}C Stand. + quercetin (10 μg)	0.86	0.85	0.85	—
^{14}C Stand. + quercetin (15 μg)	0.79	0.71	0.84	—
^{14}C Stand. + Phe (2 μg)	—	—	—	1.01
^{14}C Stand. + Phe (6 μg)	—	—	—	1.00
^{14}C Stand. + Phe (8 μg)	—	—	—	1.00

TABLE II

SCINTILLATION ACTIVITY RATIOS, OXIDIZED SAMPLES

NS = No spray; Fe = $FeCl_3$–$K_3[Fe(CN)_6]$; BDB = bis-diazotized benzidine; Nin = ninhydrin.

Sample	*Chromogenic treatment*			
	NS	*Fe*	*BDB*	*Nin*
^{14}C Standard phenylalanine (2780 dpm)	1.00	0.97	0.87	0.95
^{14}C Stand. + stilbene (2 μg)	0.99	—	—	—
^{14}C Stand. + stilbene (5 μg)	0.99	—	—	—
^{14}C Stand. + stilbene (10 μg)	0.98	—	—	—
^{14}C Stand. + quercetin (5 μg)	0.99	0.98	0.92	—
^{14}C Stand. + quercetin (10 μg)	0.99	0.98	0.91	—
^{14}C Stand. + quercetin (15 μg)	1.00	0.98	0.86	—
^{14}C Stand. + Phe (2μg)	—	—	—	1.00
^{14}C Stand. + Phe (6 μg)	—	—	—	1.00
^{14}C Stand. + Phe (8 μg)	—	—	—	1.00

TABLE III

ANALYSIS OF VARIANCE

df = Degrees of freedom; MS = mean square; NS = no spray; Fe = $FeCl_3$–$K_3[Fe(CN)_6]$; BDB = bis-diazotized benzidine; Nin = ninhydrin.

Source of estimate	*Stilbene (oxidation) NS*				*Stilbene (suspension) NS*			
	df	*MS*	*F*	$F_{0.05}$	*df*	*MS*	*F*	$F_{0.05}$
Between count rates	2	7042.5	2.2	3.98	2	21,751.9	2.5	3.98
Within count rates	11	3095.2			11	8539.0		
	Quercetin (oxidation) NS				*Quercetin (suspension) NS*			
	df	*MS*	*F*	$F_{0.05}$	*df*	*MS*	*F*	$F_{0.05}$
Between count rates	3	1666.7	0.9	3.29	3	258,429.7	137.9	3.24
Within count rates	15	1697.4			16	1873.7		
	Quercetin (oxidation) Fe				*Quercetin (suspension) Fe*			
	df	*MS*	*F*	$F_{0.05}$	*df*	*MS*	*F*	$F_{0.05}$
Between count rates	3	5157.0	2.1	3.29	3	345,995.4	51.1	3.34
Within count rates	15	2412.9			14	6776.7		
	Quercetin (oxidation) BDB				*Quercetin (suspension) BDB*			
	df	*MS*	*F*	$F_{0.05}$	*df*	*MS*	*F*	$F_{0.05}$
Between count rates	3	96,074.6	10.5	3.49	3	68,939.2	20.0	3.41
Within count rates	12	9081.6			13	3446.8		
	Phenylalanine (oxidation) Nin				*Phenylalanine (suspension) Nin*			
	df	*MS*	*F*	$F_{0.05}$	*df*	*MS*	*F*	$F_{0.05}$
Between count rates	2	5958.5	0.4	3.88	2	6654.2	1.3	3.98
Within count rates	12	13,295.5			11	4926.0		

RESULTS

The liquid scintillation counting results for suspended and oxidized samples from thin-layer cellulose chromatograms are presented in Tables I and II respectively. Statistical analysis of the samples is summarized in Table III.

DISCUSSION

With one exception, the data in Table II demonstrate that ^{14}C radioactivity labeled organic compounds definitively separated by thin-layer cellulose chromatography can be almost quantitatively measured as $^{14}CO_2$ by the direct oxidation technique described. It is further evident that the radioassay can be accomplished

independent of the structural character of the analyzed compounds or their chromogenic reaction with selective locating spray reagents. Activity ratios for the absorbed $^{14}CO_2$ were within 3% of the desired 1.00 value in four of five evaluations. The lone discrepancy observed is the case of the flavonol quercetin detected with an azo dye spray reagent. In this instance the lower activity ratios (13% at 15 μg quercetin) may be attributed to the quenching effect or competitive absorbance of the oxides of nitrogen[14] produced in the oxidation of the azo dye since a 13% decrease in activity ratio for the oxidized ^{14}C standard (non-phenolic) is observed after spraying with the azo dye. Thus, the azo dye causes a 13% reduction initially and the azo dye–phenol complex is responsible for an additional reduction in the activity ratio up to 18% at a 15 μg concentration of the phenol. The azo dye chromogenic spray reagent for phenols must then be considered unusable in conjunction with the direct oxidation technique. Fortunately, the general phenol detecting spray, $FeCl_3$–$K_3[Fe(CN)_6]$, shows near quantitative activity ratios at all concentrations of the phenol and can be used successfully with the oxidation technique.

In direct contrast to the oxidation data, Table I shows significant reductions in activity ratios for the compounds analyzed and their chromogenic spray reaction products when they are scraped from the cellulose TLC plate for direct liquid scintillation radioassay. Only phenylalanine detected with ninhydrin shows quantitative measurement of ^{14}C although the data must be corrected 12% for natural chemiluminescence of highly conjugated ninhydrin molecule. The phenols and their chromogenic spray reaction products deviate 20–30% from the desired 1.00 activity ratio.

In the case of the stilbene pinosylvin, a 20% increase in activity ratio is observed in the suspended samples. This increase is undoubtedly a direct result of the inherent chemiluminescence of the stilbene structure. In contrast, the unsprayed flavonol shows a 19% reduction in activity ratio (at 15 μg) which is due to the high optical absorptivity of the highly conjugated flavanoid nucleus. The flavonol–azo dye complex shows strong quenching with a 16% reduction in activity ratio at a 15 μg concentration. The $FeCl_3$–$K_3[Fe(CN)_6]$ sprayed flavonol shows a 29% reduction in activity ratio which must be attributed to chemical and physical quenching of the phenol–spray complex since the sprayed ^{14}C standard (non-phenolic) shows no reduction in activity ratio.

The analyses of variance presented in Table III indicate the superior precision of the oxidation techniques as applied to treated and generally chromogenically detected ($FeCl_3$–$K_3[Fe(CN)_6]$) phenolics over a concentration range of 5–15 μg. The F values for the corresponding suspension samples reflect large variations between concentrations of external standard applied. These large variations are also apparent in the calculated F values for the azo dye detected phenol in both measuring techniques. These data demonstrate the effect of the phenol and chromogenic spray–phenol complexes upon the counting results of suspended samples and further eliminates the use of an azo dye detecting reagent in either of the methods.

Analyses of variance for the ninhydrin-sprayed phenylalanine and untreated stilbene indicate no significant lack of precision for oxidation or suspension samples between external standard concentrations. However, while the suspension sample F values do reflect the precision of the recorded counting data, they do not reflect the unquantitized contribution of the previously discussed chemiluminescence on these recorded values for the stilbene and the highly conjugated ninhydrin spray.

Therefore, the analyses of variance on the suspended samples is misrepresentative. Conversely, the F values for the oxidized samples can be considered representative in the case of the stilbene and phenylalanine.

It is recognized that other techniques of elution analysis utilizing solvents to dissolve the cellulose support may result in higher recorded activity ratios for unsprayed compounds than those in this study. However, the significant reductions noted for the phenol-detecting spray complexes are independent of the chromatographic support and would most certainly affect activity ratios in any elution analysis technique. Therefore, the comparative results would conclusively indicate oxidation–absorption as a most desirable technique in radioassay of phenols requiring detection on the chromatographic support.

In addition to the contrasting counting results of Tables I and II a comparison of the sample handling characteristics of the two radioassay methods establishes some significant advantages to the direct oxidation technique, *viz.* (a) the oxidation samples are detached more cleanly and completely from the chromatographic plate by the cellulose nitrate solution, (b) the oxidation samples are not overly fragile and can be more easily transported and handled than the particulate scraped samples, and (c) the oxidation samples are unaffected by electrostatic properties of the chromatographically separated compounds or support. It is noteworthy that the greatest disadvantage of the in vial oxidation method is the restriction of sample sizes to 7 mg or less for efficient ignition. However, for large chromatographic spots, the oxidation sample may be easily divided with a razor blade after removal from the plate and oxidized in parts. The counting data accumulated for the portions can then yield the total radioactive incorporation. The oxidation method will also require the distinct separation of the radioactive components chromatographically to achieve an accurate radioassay; however, standard elution analysis methods also demand that this condition be met.

CONCLUSIONS

The results of this investigation conclusively establish that phenols and other organic compounds can be efficiently and quantitatively removed from cellulose thin-layer chromatographic plates using a nitrocellulose solution. These samples can subsequently be oxidized directly in a liquid scintillation vial to determine ^{14}C as absorbed $^{14}CO_2$. The radioactive measurements can be achieved regardless of the chemical structure or reaction products with selected chromogenic spray reagents. The method also offers superior sample preparation and handling characteristics compared to other elution analysis and oxidation methods presently in use. The technique therefore allows near quantitative determination of low level ^{14}C incorporation into phenolic or other organic components in biological or botanical feeding experiments with greater speed, higher efficiency and equal or lower cost than many other radioassay techniques presently in use.

This investigation did not consider the measurement of ^{3}H. Such a determination of ^{3}H as $^{3}H_2O$ is possible, either separately or concurrently with the measurement of $^{14}CO_2$ by the oxidation technique. The technique may also find application in the analysis of organic components on other combustible supports, *e.g.*, Sephadex or sucrose.

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REFERENCES

1 H. C. Dass and G. M. Weaver, *J. Chromatogr.*, 67 (1972) 105.
2 C. E. May and J. M. A. Brown, *J. Chromatogr.*, 53 (1970) 399.
3 J. G. Kirchner, *Thin-Layer Chromatography*, Interscience, New York, 1967. p. 177.
4 M. Wencel and K. Hoffman, *Anal. Biochem.*, 44 (1971) 97.
5 F. Snyder, in E. D. Bransome, Jr. (Editor), *The Current Status of Liquid Scintillation Counting*, Grune and Stratton, New York, 1970, p. 248.
6 A. Chakravarti and J. W. Thanassi, *Anal. Biochem.*, 40 (1971) 484.
7 E. D. Bransome Jr. and M. F. Grower, *Anal. Biochem.*, 38 (1970) 401.
8 L. A. Wegner and H. Winkelman, *Atompraxis*, 16 (1970) 19.
9 G. N. Gupta, *Anal. Chem.*, 38 (1966) 1356.
10 N. Kaartinen, *Packard Techn. Bull.*, No. 18, Packard Instr. Co., Downers Grove, Ill., Dec. 1972.
11 D. G. Roux and A. E. Maihs, *J. Chromatogr.*, 4 (1960) 65.
12 T. K. Bell, *Lab. Pract.*, 17 (1960) 609.
13 C. A. Bennett and N. L. Franklin, *Statistical Analysis in Chemistry and the Chemical Industry*, Wiley, New York, 1954, p. 319.
14 V. V. Kerr, F. N. Hayes and D. G. Otto, *Int. J. Appl. Radiat. Isotop.*, 1 (1957) 284.