Qualitation and Quantitation of Some Terphenylquinones

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Microchemical and photochemical procedures which would permit rapid, sensitive detection of atromentin, aurantiacin, and thelephoric acid were examined. Photochemically induced fluorescence and chromophore formation with a ceric ammonium sulfate (CAS) reagent were useful for this purpose, especially when the terphenylquinones were adsorbed on polyamide powder. Quantitation of atromentin was possible with a CAS procedure, and all three of the terphenylquinones could be quantitated with pyridine–water (Py–W) and combined Py–W–CAS procedures. The relative sensitivities for the quantitative methods for atromentin were CAS > Py–W–CAS > Py–W. The procedures permitted the determination of atromentin and aurantiacin in concentrations of 2 to 120 mcg./ml. and 4 to 120 mcg./ml., respectively, and of thelephoric acid in concentrations of 2 to 40 mcg./ml. Precise standardization of analytical conditions was critical.

QUINONES comprise a highly reactive, heterogeneous group of compounds which are attracting increasing interest as basic research tools in biochemistry. Quinones and compounds capable of forming quinoid-like structures are strongly conjugated chemical systems which have the capacity for rapid electronic delocalization. This important molecular feature is of potential biochemical significance in energy transfer, long-range transmission of electronic excitation, and resonance stabilization of activated complexes (1). Molecular biology and pharmacology (2, 3), the molecular orbital approach to quantum chemistry (1, 4), photochemistry of organic compounds (5–7), and oxidation-reduction mechanisms in polymers (8) are examples of other areas where study of quinoid systems is useful.

When information about the quinoid compounds is more complete, application of this knowledge will permit elucidation of the mechanisms of action of many drugs and will improve their utilization in therapy. Webb (3) has emphasized the medicinal potential for biologically active quinones by reviewing their use as antitumor or antimitotic agents, as enzyme inhibitors, as possible acceptors in electron transport systems, as uncouplers of oxidative phosphorylation, and as antimicrobial agents. Other interesting functions of quinones in biologic systems include radiosensitizers in tumor therapy (9), antioxidants (10), and storage of solar energy (11).

Terphenylquinones are a group of naturally occurring pigments about which very little is known. The available knowledge of this group of quinones is restricted to elucidation of their chemical structures, distribution of certain of the constituents in a limited sampling of fungi and lichens (12, 13), and gross observations of the antitumor activity of polyporic acid (14, 15) and the anticoagulant action of atromentin (16, 17). There is a paucity of information on many of the chemical properties of these compounds, and information on the biologic involvement of these constituents at the molecular level is totally lacking for both plant and animal systems.

Studies were undertaken to develop sensitive procedures for qualitative and quantitative determination of atromentin, aurantiacin, and thelephoric acid. The availability of such analytical techniques would facilitate investigations on the role of these quinones in biologic systems. A secondary objective of the study was accumulation of data on the chemical properties of the three terphenylquinones.

EXPERIMENTAL

Materials and Reagents—Atromentin, aurantiacin, and thelephoric acid were isolated from suitable fungi using procedures which had been tested in this laboratory (13, 16, 18). Purity of the isolated compounds was verified using melting points, elemental analyses, and absorption spectroscopy (infrared and ultraviolet). Eastman spectrograde methanol and pyridine were used routinely when spectroscopic manipulations were involved. The 1% ceric ammonium sulfate (CAS) reagent in syrupy *o*-phosphoric acid was prepared as described by Farnsworth *et al.* (19) and was stored in a tightly stoppered actinic glass flask. The undiluted CAS reagent was used as a spray for detection of the terphenylquinones on the thin-layer chromatograms, but the reagent was diluted with an equal volume of distilled water immediately prior to its use in quantitative procedures.

General Quantitative Methodology—Preliminary results indicated that daily preparations of standard solutions of the terphenylquinones and storage

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TABLE I—ABSORPTION MAXIMA OF THELEPHORIC ACID IN AQUEOUS PYRIDINE MIXTURES

Vol. of Pyridine, ml.	Vol. of Water, ml.	Absorption Max., mμ
3	0.5	525,340
3	0.75	540,338
3	1.0	552,338
3	2.0	640,338

of the solutions in tightly stoppered actinic glass containers were essential for reproducibility. Standard stock solutions were prepared by dissolving 120 mcg./ml. of atromentin or aurantiacin or 40 mcg./ml. of thelephoric acid in pyridine or 10 mcg./ml. of atromentin in an absolute methanol–glacial acetic acid mixture (99:1) using actinic glass volumetric flasks. These concentrations were selected when preliminary evidence indicated that they were near the upper limits which gave reproducible results. Five dilutions were made from each stock solution, and samples of these solutions were utilized in developing the quantitative procedures. The visible spectra of the colored solutions were scanned using a Beckman recording spectrophotometer, model DB, to determine absorption maxima. A corresponding reagent blank was routinely employed in all spectrophotometric examinations. Studies were also conducted to determine the time which was required for maximum color formation and to show the relative stability of the colored complex. After suitable absorption maxima were selected and optimal experimental conditions were determined, standard curves were prepared and molecular extinction coefficients were calculated using average data from triplicate quantitations. The standard curves were plotted on the bases of quinone concentrations in the pyridine or methanol solutions, but allowances were made for the volume of additives in calculating the molecular extinction coefficients of the colored complexes.

Pyridine–Water (Py–W) Colorimetric Procedure—Sawada (20) qualitatively utilized the color change from a grape-red to blue when water was added to a pyridine solution of thelephoric acid. Variations of this approach were found to give quantitative results with atromentin, aurantiacin, and thelephoric acid. The quantity of distilled water which was added to the pyridine solutions of atromentin and aurantiacin had no significant qualitative influence on the color, but the absorption maxima of the colored complex varied when different amounts of water were added to the thelephoric acid solutions (Table I). A standard technique utilizing 3 ml. of a terphenylquinone-containing pyridine solution and 0.75 ml. of water was selected as being feasible. The absorbance of the atromentin solution was measured at 560 mμ 15 to 30 min. after thorough mixing. Absorbance of the thelephoric acid colored complex was measured within 2 to 35 min. at 540 mμ. (Fig. 1.)

Color formation with aurantiacin was very slow to develop at room temperature, and difficulty was noted in obtaining reproducible color intensities. This was presumably caused by the need for hydrolysis of the benzoate esters of aurantiacin to yield atromentin prior to the formation of the colored complex. Satisfactory quantitative results were obtained when the aurantiacin mixture was heated initially. The aqueous pyridine solution was placed in an actinic glass test tube which was covered with a marble to prevent evaporation, the solution was heated in a steam bath for 10 min., and the mixture was cooled rapidly by immersing the tube in water at room temperature. Absorbance was measured at 560 mμ within 30 min. (Fig. 1).

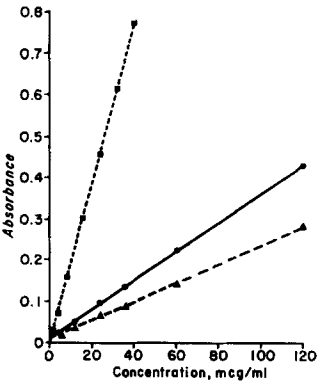

Fig. 1—Quantitation of atromentin, aurantiacin, and thelephoric acid with the Py–W procedure. Key: ●, atromentin, 560 mμ; ▲, aurantiacin, 560 mμ; ■, thelephoric acid, 560 mμ.

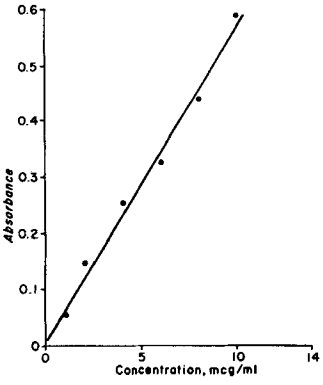

Fig. 2—Quantitation of atromentin at 418 mμ with the CAS procedure.

Reproducibility among the triplicate determinations was excellent, varying no more than ±0.005 absorbance units for any tested concentrations of the terphenylquinones.

CAS Colorimetric Procedure—CAS has been used to measure oxidation-reduction potentials of some quinones (21), and the reagent has been shown to have unusual utility in the chromatographic determination of *Catharanthus* alkaloids (19). These applications suggested the possibility of this reagent for the qualitative or quantitative evaluation of the terphenylquinones. A ceric hydroxide precipitate forms in alkaline solutions, and it was considered desirable to use methanol rather than pyridine to dissolve the pigments. A methanol–glacial acetic acid mixture (99:1) was selected after preliminary results with atromentin revealed that the small quantity of acetic acid did not effect quantitation but did greatly facilitate elution from thin-layer chromatograms. Atromentin could be quantitated by adding 0.04 ml. of the 0.5% CAS reagent to 4 ml. of the methanolic solutions using a 0.1-ml. pipet

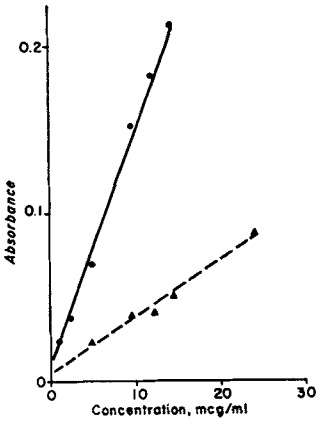

Fig. 3—Quantitation of atromentin and aurantiacin with the Py – W – CAS procedure. Key: ●, atromentin, 525 mμ; ▲, aurantiacin, 500 mμ.

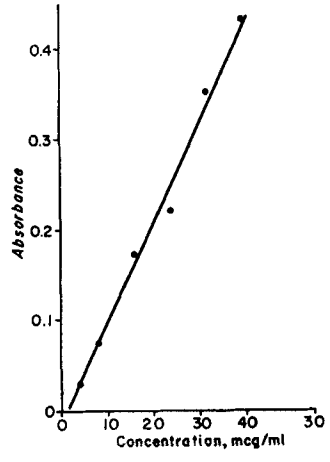

Fig. 4—Quantitation of thelephoric acid at 500 mμ with the Py–W–CAS procedure.

calibrated in 0.002-ml. increments, mixing vigorously several times during a 30-min. reaction period, and measuring the absorbance of the bright yellow color at 418 mμ (Fig. 2). Background absorption with the CAS reagent necessitated particularly careful preparation of the blank.

Reproducibility among the triplicate determinations was approximately ±3%. The procedure had a sensitivity for atromentin that was more than 15 times that of the Py–W procedure. However, the CAS reagent could not be used to quantitate aurantiacin and thelephoric acid. Thelephoric acid is not appreciably soluble in nonbasic organic solvents, and no suitable method was found to hydrolyze aurantiacin without interfering with chromophore formation.

Pyridine–Water–Ceric Ammonium Sulfate (Py–W–CAS) Colorimetric Procedure—The Py–W procedure had the advantage of applicability to all three of the terphenylquinones which were studied, but the advantageous feature of the CAS procedure was increased sensitivity. The possibility of combining these two advantages was explored, and preliminary results revealed that quantitation was possible with dilute solutions of the quinones. Several different combinations of conditions and reagents were observed to give reproducible quantitative results, but the procedure which was selected as being most useful involved the initial measurement of the terphenylquinones using the previously described Py–W procedure. After reading the absorbance of an aqueous pyridine solution, it was transferred quantitatively from the cell to an actinic glass test tube with the aid of 3.75 ml. of methanol, a component which was found to be essential for the subsequent quantitation, and 0.04 ml. of 0.5% CAS reagent was added. The solution was mixed thoroughly, and the color was allowed to develop for 1 hr. Absorbance of the colored complex was measured at 525 mμ for atromentin and at 500 mμ for aurantiacin and thelephoric acid (Figs. 3 and 4).

Reproducibility among the triplicate determinations was equivalent to that obtained with the Py–W procedure. The combined procedure was more sensitive than the Py–W method for atromentin and aurantiacin, but when allowance was made for volume change, the sensitivities of the two procedures were comparable for thelephoric acid. The combined method was approximately half as sensitive as the CAS procedure for atromentin.

Qualitative Determinations—Characteristic absorption maxima (Table II) and other individualistic properties which were observed during development of the quantitative procedures were useful in the qualitative evaluation of the three terphenylquinones. It was also noted that these terphenylquinones were susceptible to oxidative and photochemical changes, properties which are well documented for other quinones. This instability limited the types of manipulations which could be utilized in qualitative verification of small quantities of these pigments, and studies were undertaken to discover additional methods which would contribute to rapid identification. It was considered desirable to include a chromatographic procedure in the qualitative evaluation to permit examination of mixtures and to provide a possible basis for subsequent quantitation of impure materials or components of mixtures.

TABLE II—ABSORPTION MAXIMA AND MOLECULAR EXTINCTION COEFFICIENTS OF ATROMENTIN, AURANTIACIN, AND THELEPHORIC ACID AND THEIR COLORED COMPLEXES

Compd.	Untreated Soln. Solvent	mμ	log ε	Complex with Py–W mμ	log ε	Complex with CAS mμ	log ε	Complex with Py–W–CAS mμ	log ε
Atromentin	Dioxane	268	4.58	340	3.96	418	4.30	525	4.09
		385	3.75	560	3.16				
Aurantiacin	Dioxane	240	4.63	330	3.98	...	...	352	3.87
		405	3.92	560	3.20			500	3.61
Thelephoric acid	Pyridine	311	4.51	338	4.35	...	...	500	3.53
		495	4.11	540	3.95				

TABLE III—QUALITATIVE EVALUATION OF ATROMENTIN, AURANTIACIN, AND THELEPHORIC ACID USING POLYAMIDE THIN-LAYER CHROMATOGRAPHY[a]

Compd.	R_f	Color[b]	Color with CAS Spray[b]
Atromentin	0.13–0.25[c]	10dc, orchid haze	2 min.: 23ic, apple green 15 min.: 1kb, light citron yellow 30 min.: 2kb, golden yellow
Aurantiacin	0.80	2fb, straw wheat	2fb, straw wheat
Thelephoric acid	0.05	8ec, rose mist	9ic, raspberry rose

[a] Methanol–chloroform–water–glacial acetic acid (54:36:6:1) solvent system. [b] Color chip number and trivial name from "Color Harmony Manual." [c] Migration of atromentin was observed to be concentration dependent. Larger quantities of the quinone, up to the point where the system became overloaded, showed greater movement.

Preliminary results revealed that columnar and paper partition chromatographic separations were too slow for any evaluations of these compounds involving ultimate quantitation. Elution of the terphenylquinones following their separation with an established thin-layer chromatographic method (13) was inefficient, and the solvent interfered with quantitative attempts. Thin-layer chromatography with polyamide was found to be useful qualitatively and to offer quantitative possibilities. When the polyamide chromatograms were prepared without a binder, extreme care was necessary to avoid mechanical loss in removing spots of the brittle film from the chromatograms for quantitative purposes. The other extreme was noted with a modified formulation of the adsorbent (22) where adherence to the plates made quantitative applications impractical. The most satisfactory results were obtained with a modification employing one-fourth the rice-starch binder recommended by Nordby *et al.* (22). Rice starch (0.2 Gm.), silica gel without binder (Woelm, 0.1 Gm.), and water (9 ml.) were heated in a covered beaker on a steam bath for 40 min. with frequent stirring. The binder was transferred, with the aid of 3 ml. of water, to a Waring blender containing 5.5 Gm. of Woelm polyamide TLP and 30 ml. of methanol, and the mixture was blended for 5 min. at high speed. The slurry was applied to glass plates with a chromatographic spreader; the plates were allowed to air-dry for 15 min. and were placed in a desiccator over anhydrous $CaCl_2$ for a minimum of 2 hr. before use. Several chromatographic solvent systems effected separation, but some tailing was always noted with atromentin, presumably due to a pronounced tendency for hydrogen bonding. Methanol–chloroform–water–glacial acetic acid (54:36:6:1) was selected as the best solvent for combined qualitative and quantitative objectives; higher proportions of water or acetic acid enhanced the instability of the terphenylquinones. The chromatograms were placed in the dark at room temperature for 15 min. to permit the solvent to evaporate prior to qualitative or quantitative evaluation.

The distinctive colors of the terphenylquinones were recorded by direct comparison with chips from a "Color Harmony Manual" (23, 24), and the chromatograms were subjected to further evaluations. Particularly characteristic results were observed with atromentin when chromatograms were sprayed lightly with 1% CAS reagent. The chromatograms became transparent when sprayed, but the complexes could be visualized by briefly layering the adsorbent with a film of water to make the background opaque. The initial colors were relatively stable with aurantiacin and thelephoric acid, whereas a characteristic color sequence developed with atromentin (Table III).

None of the terphenylquinones fluoresced upon initial examination of the chromatograms under U.V. light, but fluorescence was observed in solutions of the compounds which had undergone photochemical changes while stored in tightly stoppered Pyrex glass containers. Unsprayed chromatograms were exposed to a Hanovia high pressure quartz mercury vapor lamp, model 30600, at approximately 15 cm. for 24 hr.; this radiation produced no detectable changes with thelephoric acid, but a yellow fluorescence was noted with atromentin and aurantiacin.

The qualitative fluorescent properties of the photoproducts of the terphenylquinones were examined further by radiating methanolic solutions of the compounds and determining their excitation and emission peaks with a Baird Atomic Fluorispec, model SF-1. Solutions containing 20 mcg./ml. of atromentin or aurantiacin or 10 mcg./ml. of thelephoric acid were prepared and radiated in Pyrex glass volumetric flasks with the mercury vapor lamp. The fluorescent properties of the photoproducts were determined after radiating atromentin and aurantiacin for 5 days and thelephoric acid for 10 days (Table IV).

Quantitation of Chromatographically Separated Terphenylquinones—Studies with pure terphenylquinones revealed that semiquantitative to quantitative results could be achieved by applying the various colorimetric procedures following thin-layer chromatographic separation. Appropriate zones of the polyamide adsorbent were transferred to actinic glass test tubes as rapidly as possible, and the colorimetric reagents were added to the tubes. The colors were allowed to develop for the

TABLE IV—QUALITATIVE FLUORESCENT PROPERTIES OF PHOTOPRODUCTS FROM ATROMENTIN, AURANTIACIN, AND THELEPHORIC ACID

Compd.	Excitation, mμ	Emission, mμ
Atromentin	365	415 525
	395	525
Aurantiacin	365	485
	400	515
Thelephoric acid	370	415 495
	397	502

previously indicated periods of time with frequent agitation, the polyamide powder was removed by filtration, and the absorbances were measured spectrophotometrically at the appropriate wavelength.

The Py–W–CAS procedure was found to be the method of choice for atromentin and aurantiacin. In the case of aurantiacin, the aqueous pyridine solution was subjected to the hydrolytic conditions prior to addition of the methanol and CAS reagent. Since the total volume of liquid with this procedure was greater than that required to fill the cell, the sensitivity could be doubled by utilizing only half the specified volume of each component in the reaction mixture. The more complex Py–W–CAS procedure offered no sensitivity advantage over the Py–W method for thelephoric acid, so the latter was utilized. Acceptable quantitation of atromentin was possible upon application of the CAS colorimetric procedure, but the method had little apparent practicality.

Combination of any of the colorimetric procedures with chromatography resulted in the expected losses due to transfer and elution of the adsorbent, but the most critical factor was found to be losses through oxidative and photochemical changes if the manipulations were not handled rapidly. The best results were obtained with the relatively stable thelephoric acid. When chromatograms were exposed to air and light for 2 hr. before evaluation, losses of atromentin were 30% or more. Tailing of atromentin on the chromatograms sometimes further complicated quantitations, and occasionally inexplicable results were obtained following hydrolysis of aurantiacin in the presence of the polyamide powder. However, under optimal conditions the colorimetric results were consistently ±5–10% of the theoretical values.

DISCUSSION

Three rapid and sensitive colorimetric procedures were developed for the quantitation of atromentin, aurantiacin, and thelephoric acid (Figs. 1–4). Reproducibility, both within replicate determinations and with analyses conducted at different times, required strict adherence to precautionary measures to minimize oxidative and photochemical changes. Photochemical changes could be controlled during some of the manipulations by utilizing actinic glassware. However, oxidative interference still necessitated completion of the analyses in the minimum possible time.

Quinones are known to be susceptible to such photochemically induced reactions as fragmentation, polymerization or addition of other available substituents, rearrangement, and reduction (5, 7). The exact nature of the photochemical reaction(s) with the terphenylquinones was not determined due to the difficulty in distinguishing between oxidative and photochemical changes. The shift in fluorescent characteristics when solutions were radiated for varying periods of time suggested the solutions contained mixtures of transformation products. Atromentin was the least stable of the three terphenylquinones, and observations on the changes with this pigment were of the greatest indicative value.

The transformation products were observed to cause two types of problems in the quantitative procedures. These substances did not form the characteristic colored complexes, an observation which was substantiated by adding water to a radiated pyridine solution of atromentin and noting that the absorption maximum at 560 mμ was missing (the absorption peak at 340 mμ remained). The second problem suggested that polymerization was at least one of the photochemical changes. After cells had been used for several analyses, fluorescent interference was noted in the ultraviolet region. It was determined that the interference was caused by the deposition of minute amounts of insoluble photoproducts on the walls of the cells. The problem was noted initially during studies where the absorbance of a solution was read periodically to determine the time interval which gave quantitative results, but subsequent studies showed that the effect was cumulative when a succession of freshly prepared solutions were allowed to remain in the cell for a few minutes each. The deposit could be removed either with alcoholic KOH or dichromate cleaning solution, but it was insoluble in the normal organic solvents. The insolubility of the product suggested it was polymeric, thus making this problem similar to that reported by Pitts *et al.* (25).

Finite characterization of the chromophores in the various colorimetric procedures was not possible with the available data, but observations suggested some of the apparent prerequisite features of the terphenylquinones. The most important molecular feature appeared to be the 3,6-dihydroxy substituents. Aurantiacin was presumably hydrolyzed to atromentin prior to formation of the chromophore in the Py–W quantitation. This presumption was supported by the failure of aurantiacin to form a colored complex immediately and by the observation that the ratio of absorbances of the colored complexes formed with equal quantities of atromentin and aurantiacin was 3:5, a ratio approximately that of their molecular weights. The predicted involvement of the 3,6-dihydroxy groups was consistent with the observed failure of 2,5-diphenyl-1,4-benzoquinone to form a chromophore in the Py–W system. Consideration of the importance of the hydroxyl substituents and the conjugated nature of these terphenylquinones suggested the chromophore in Py–W was probably a resonance hybrid of the parent quinone.

Resonance hybridization also appeared to explain the chromophores which were formed when the terphenylquinones were chromatographed on polyamide powder. The chromophores which were formed on the polyamide chromatograms did not interfere with subsequent chromophore formation in the colorimetric procedures when the quinones were eluted, thus indicating that chemical change was unlikely. The possibility that the nitrogen atoms in the adsorbent stabilize the resonance hybrid, presumably by sharing protons, was further suggested when comparable chromophores were noted upon the addition of the terphenylquinones to solutions of gelatin. It should be noted that the chromophore with thelephoric acid and polyamide or gelatin was greenish turning to pink upon addition of small quantities of acetic acid.

CAS is an oxidizing agent, and chromophores developed with the terphenylquinones and this

reagent appeared to involve irreversible chemical change. Attempts to determine the oxidation-reduction potentials of the terphenylquinones with CAS were unsuccessful, but the availability of this reagent for reaction with the quinones was found to be the limiting factor in the CAS and Py–W–CAS colorimetric procedures. CAS had limited solubility in the solvents which were employed in these methods, and quantitative chromophore formation was limited by this factor. The solubility of CAS in the solvent mixture of the Py–W–CAS procedure was greater than in the methanolic solvent of the CAS method. However, attempts to determine solvent mixtures which permitted higher concentrations of CAS encountered problems with the solubilities of the terphenylquinones or with the fundamental involvement of methanol in the chromophore-yielding reactions. Addition of CAS to an aqueous pyridine solution of atromentin resulted in a loss of the absorption maximum at 560 mμ, but formation of a characteristic new chromophore with peak absorption at 525 mμ was observed only when methanol was in the reaction mixture.

Qualitative data suggested that thelephoric acid did not react with CAS. Addition of methanol and the CAS reagent to an aqueous pyridine solution appeared merely to dilute the colored complex; the color intensity could be predicted by correcting for volume change. Absorption maximum of the thelephoric acid complex with the Py–W–CAS method was 500 mμ, but this shift was attributed to the known qualitative influence of additives on the chromophore of this quinone in pyridine solutions (Table I). The observation that methanol was not an essential component of reaction mixtures containing CAS and thelephoric acid, in contrast to mixtures involving atromentin and aurantiacin, was consistent with the presumed lack of chromophore formation with thelephoric acid, the most stable of the three terphenylquinones.

Preliminary results revealed that the colorimetric methods were suitable for proximate quantitations of pure terphenylquinones following thin-layer chromatography. This suggests the possibility of devising conditions which are suitable for quantitative evaluation of the terphenylquinones in fungi or other biologic systems. Application of some type of rapid analysis appears mandatory to obtain reasonable quantitation of these reactive compounds, but it will be necessary to check each new condition or system to exclude possible interference which may be caused by the new condition or by other components in the biologic system.

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