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Note

Determination of rotenone in pesticide formulations and the separation of six rotenoids by reversed-phase high-performance liquid chromatography

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The analysis of rotenone in pesticide formulations is very complex due to the presence of other rotenoids and frequently additional pesticides which are co-formulated with rotenone[1,2]. Rotenone is often the least concentrated pesticide in mixed formulations; thus, the difficulty of analysis is compounded by high concentrations of interfering pesticides. Recently two methods, one employing gas–liquid chromatography (GLC)[3] and the other using high-performance liquid chromatography (HPLC)[2], have been developed which eliminate interfering compounds. Both methods are rapid, specific, and appear to exclude all interferences in the quantitation of rotenone.

The previously reported HPLC method[2] employs normal-phase chromatography on silica. We have developed another HPLC method using reversed-phase chromatography. The new method has the advantages of faster analysis, separation of additional rotenoids, and a more stable column. The results of this research, including analysis of six commercial products and the separation of various rotenoids, are given.

EXPERIMENTAL

Materials and equipment

Rotenone with a purity of 99% was obtained from Aldrich (Milwaukee, Wisc., U.S.A.) whereas all other rotenoids were obtained from Martin Jacabson (United States Department of Agriculture, AEQ 1 Biologically Active Natural Products Laboratory, 323 South, ARC Bldg., 306 Beltsville, Md., U.S.A.). The extracting solvent, dioxane, was analytical reagent obtained from Mallinckrodt (St. Louis, Mo., U.S.A.).

The high-performance liquid chromatograph utilized was from Waters Assoc. (Milford, Mass., U.S.A.), and included a Model 6000 A solvent delivery system, a Model 440 absorbance detector, and a U6K septumless injector. The detector was set on 280 nm and a sensitivity of 0.5 a.u.f.s. when analyzing formulations and 0.1 a.u.f.s. for the separation of rotenoids. Connected to the detector was a Rikadenki recorder (Rikadenki Soltec, Encino, Calif., U.S.A.), which was set on 10 mV and at a chart speed of 20 cm/h for formulation analysis or 10 cm/h for the separation

of rotenoids. A two-column system was used: a 4 in. × 1/8 in. O.D. stainless-steel precolumn packed with C_{18} Corasil (Waters Assoc.; 37–50 μm) followed by a 30 cm × 4 mm I.D. column of specially drilled stainless steel packed with μBondapak/C_{18} (Waters Assoc.). Both columns were maintained at ambient temperature.

Methods

The analysis and preparation of rotenone formulations were divided into two classes: dusts and liquids. Dust samples, equivalent to 20 mg of rotenone, were weighed into 125-ml glass-stoppered erlenmeyer flasks and extracted with 50 ml of dioxane on a gyratory shaker for 1.5 h. After shaking, the samples were filtered through 15 cm Whatman No. 2 fluted paper directly into 50-ml beakers. Approximately 10 ml of the filtrate were refiltered through an organic sample clarification kit (Waters Assoc.) containing 0.5-μm filters. The samples were then injected (10 μl) into the HPLC apparatus. The solvent system was methanol–distilled water (80:20) with a flow-rate of 1 ml/min. A rotenone standard (20 mg/50 ml) was first injected (10 μl) followed by two injections of sample (10 μl) and finally another injection of standard.

Liquid samples, equivalent to 10 mg of rotenone, were weighed into 25-ml volumetric flasks and brought to volume with dioxane. These were also filtered through the clarification kit before injecting. The injection volume and operating conditions were the same as for the dust samples.

To show that several different rotenoids could be separated using reversed-phase, standards of each were weighed at a concentration of 0.2 mg/ml (except for deguelin, 0.5 mg/ml) and injected at a volume of 20 μl. The solvent was methanol–distilled water (60:40) with a flow-rate of 2 ml/min.

Calculations

Peak height were used to determine the amount of rotenone in the formulations. Once the peak heights of the standard and sample had been measured and averaged, they were substituted into the formula below:

$$\% \text{ Rotenone} = (Hu/Hs) \times (Ws/Wu) \times \% \text{ purity of standard}$$

where *Hu* and *Hs* are average peak heights of the sample and standard, respectively, *Ws* is the grams of rotenone standard/50 ml, and *Wu* is the grams of sample extracted.

RESULTS AND DISCUSSION

A recovery study was performed by spiking six flasks. Each flask contained 3 g of rotenone-free dust. One group of three was spiked with 20 mg, while the other group had 50 mg added. The results of the study ranged from 93.8–96.2% recovery with a mean of 94.3% for the 20-mg set and 95.4% for the other group.

The linearity of peak heights was also tested, which showed that they are linear from a few ng to 14 μg, provided that a volume not greater than 12 μl was injected. When volumes of more than 12 μl were injected, the plot of mass *versus* peak height became biphasic (Fig. 1). The retention time (R_t) for the leading edge of the rotenone

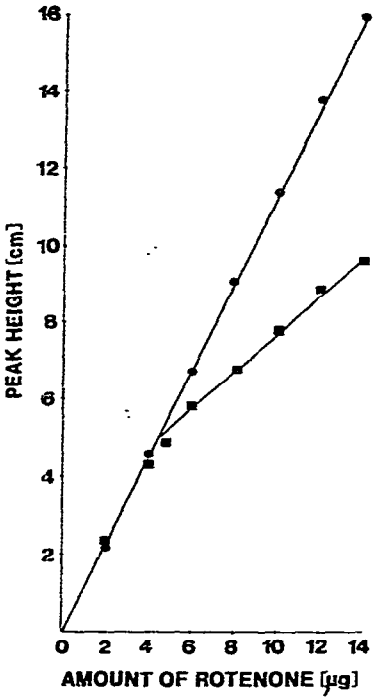

Fig. 1. Graph of peak height *vs.* amount of rotenone, where ● represents the plot of constant volume injection (5 µl) and ■ represents the plot of varying volume injections (5–35 µl). Dioxane was the injecting solvent.

peak was not affected at injection volumes greater than 12 µl, but the R_t of the trailing edge increased. Thus, the peak width increased and the peak height decreased. The R_t phenomenon has been described by Scott and Kucera[4] in a study of the parameters of preparative-scale liquid chromatography. Apparently the larger volumes of solvent, dioxane, cause column "overload" when injected into the eluting solvent, methanol–water (80:20). We first suspected that the overload occurred on the low-capacity pre-column; however, removal of the precolumn did not eliminate the overload condition. Thus, we conclude that the problem is most probably "volume overload"[4].

The results of six analyses of commercial formulations are given in Table I. Generally, the per cent rotenone found by this method is lower than the guarantee. This was also observed in our previous HPLC study, at which time we did a comparison of the HPLC method and the ultraviolet (UV) procedure previously accepted by the Association of Official Analytical Chemists (AOAC)[5]. It was observed that the UV method gave a high bias because of interfering rotenoids that are oxidative products of rotenone. Delfel[1] has recently conducted an extensive study on these interferences *vs.* the UV, Infrared (IR), and crystallization methods of the AOAC and has recommended that the GLC and HPLC methods be evaluated for possible adoption as the official AOAC method for rotenone determination.

A typical chromatogram of rotenone and a commercial formulation containing rotenone can be seen in Fig. 2. Rotenone will elute in approximately 6 min under these conditions, with 6$\alpha\beta$,12$\alpha\beta$-rotenolone and deguelin being separated from

TABLE I

DETERMINATION OF ROTENONE IN COMMERCIAL DUST AND LIQUID FORMULATIONS BY HPLC

*Sample**	*Dust*	
	Label guarantee (%)	*Rotenone found (%)*
1	5.0	3.04**
2	5.0	4.71
3	0.75	0.70
4	0.75	0.77
5	1.0	0.62
	*Liquid****	
	Label guarantee (%)	*Rotenone found (%)*
6	0.128	0.095

* Samples were analyzed in triplicate.
** Means of the three analyses.
*** Aerosol.

rotenone with only a 10% overlap. Tephrosin will co-chromatograph with rotenone but does not appear to give a high bias since analyses of some of the formulations using the normal-phase HPLC method which separates tephrosin from rotenone give good agreement between results obtained by reversed-phase HPLC. The absorbance of tephrosin ($\lambda_{max} \approx 270$ nm) in methanol is approximately 40 times less than that of rotenone ($\lambda_{max} \approx 294$ nm) when compared at 280 nm. Also tephrosin is present in formulations at low levels —occasionally about 5% of rotenone concentration with a maximum of 10% and frequently not detectable. Thus, the maximum tephrosin level we have observed in the analysis of formulations containing rotenone would produce only a 0.25% high bias in actual rotenone content by analysis using reversed-phase chromatography.

Rotenone is formulated with numerous other pesticides of which the most common are: karathane, folpet, captan, MGK 264, piperonyl butoxide, pyrethrins, methoxychlor, diclone, and carbaryl. Of these diclone, MGK 264, and captan co-chromatograph with rotenone but MGK 264 and captan only weakly absorb at 280 nm, whereas diclone absorbs extensively. With samples containing diclone the solvent system acetonitrile–distilled water (60:40) with a flow-rate of 1 ml/min can be used.

Fig. 3 shows a liquid chromatogram obtained when a mixture of rotenoids (tephrosin, 6$\alpha\beta$,12$a\beta$-rotenolone, deguelin, rotenone, dehydrorotenone, and β-dihydrorotenone) was injected. The entire separation took 30 min, with all six compounds being separated. The separation between the rotenoids was not baseline; however, baseline separation can be achieved by using methanol–distilled water (55:45) at a flow-rate of 2 ml/min. The entire separation takes 80 min. The elution of these rotenoids on reversed phase is almost the complete reversal of the normal-phase

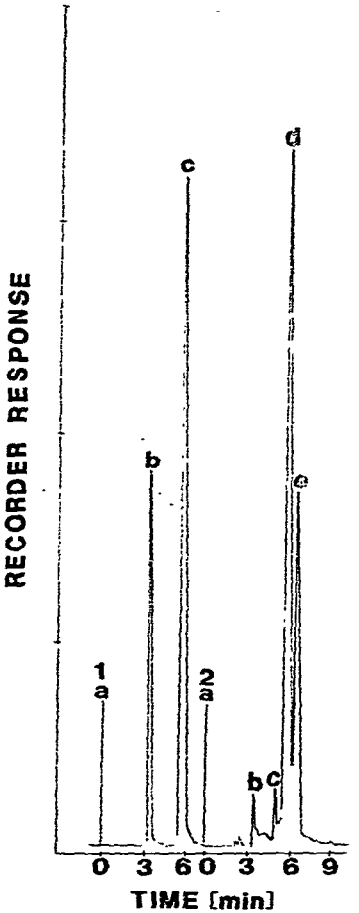

Fig. 2. Liquid chromatograms of rotenone. (1) Analytical-grade rotenone: a = point of injection; b = solvent peak; c = rotenone (4 μg/10 μl). (2) Commercial formulation of rotenone: a = point of injection; b = solvent peak; c = 6$\alpha\beta$,12$\alpha\beta$-rotenone; d = rotenone and tephrosin; e = deguelin. See Methods for operating conditions.

elution order, with the exception of β-dihydrorotenone. On normal phase, the peaks elute as follows: dehydrorotenone, deguelin, rotenone, β-dihydrorotenone, and 6$\alpha\beta$,12$\alpha\beta$-rotenolone and tephrosin cochromatographing.

The limits of detection of rotenone were tested. It was observed that this method could readily be applicable for residue analysis since 2 ng could be detected using an attenuation of 0.005 a.u.f.s.

This reversed-phase HPLC method of analysis for rotenone is preferred over the silica gel HPLC procedure, since analyses can be completed in less time with better separation and equal precision. Also, our experience in analyzing pesticide formulations by HPLC shows reversed-phase columns to be more stable, to reach equilibrium faster, are easy to clean, and have a longer lifetime than silica gel columns.

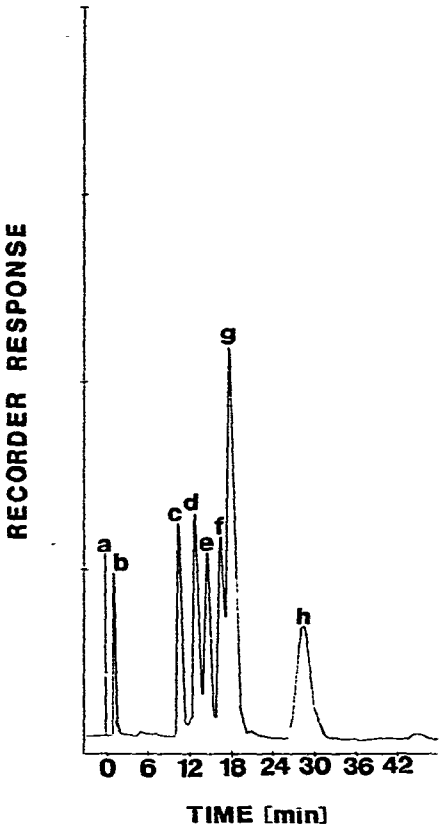

Fig. 3. Liquid chromatogram of 20 μl mixed rotenone solution. a = Point of injection; b = solvent peak; c = 6$\alpha\beta$,12$\alpha\beta$-rotenolone; d = tephrosin; e = rotenone; f = β-dihydrorotenone; g = deguelin; h = dehydrorotenone. See Methods for operating conditions.

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