Distribution of Rotenone and Deguelin in *Tephrosia vogelii* and Separation of Rotenoid-Rich Fractions

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Rotenone and deguelin were determined by thin-layer densitometry in seven varieties of *Tephrosia vogelii*. The leaflets contained more deguelin than rotenone; the reverse was generally found in the petioles, stems, and roots. One variety contained deguelin but no rotenone. In other varieties the leaflets had more rotenone than other parts of the plant, and several times as much deguelin. Fractionation to recover rotenoid-rich leaflets was pos ible because they were more friable than stem portions of partially dried plants. Minimizing drying reduced rotenoid destruction. Separation by a whizzer-type air classifier was preferred over hammer or disc mills followed by sieving for three reasons: it was operable with higher moisture material (leaflet moisture content 12–14%), so rotenoid loss during drying was reduced; it avoided additional losses possibly caused by frictional heating in the mills; and it gave virtually complete (up to 97%) separation of leaflets from stem-petiole fractions.

An annual legume *Tephrosia vogelii* Hook. f. (Figure 1A) is being domesticated by the U.S. Department of Agriculture (Barnes *et al.*, 1967) as a source of the natural insecticides and fish poisons, rotenone and deguelin. For practical purposes these two rotenoids together make up the total rotenoid content of this plant (other rotenoids being present in only minor amounts). Because a suitable analytical method has not been available, the individual distribution of rotenone and deguelin within the *T. vogelii* plant has not been studied, but Worsley (1939) and Irvine and Freyre (1959) have reported the total rotenoid distribution based on non-specific color tests. Unlike the present commercial sources of rotenone, *T. vogelii* deposits most of its rotenoids in its above ground parts, a distinct advantage for machine harvesting. The concentration of rotenoids is much higher in the leaflet than in other parts of the plant, however, and separation of leaflets would be desirable to reduce extraction costs. In buckwheat (Phillips *et al.*, 1950) and in alfalfa (Chrisman and Kohler, 1964) leaf-stem separation has been achieved after differential milling of the partially dried plant (dry leaves, moist stem).

A thin-layer densitometric analysis was recently developed for rotenone and deguelin (Delfel and Tallent, 1969). One objective of the present investigation was to use this procedure to determine the individual distributions of rotenone and deguelin within *T. vogelii* plants. A second objective was to produce from harvested plants a fraction with maximum concentration of these rotenoids, thereby minimizing extraction costs.

EXPERIMENTAL

Plant Material. Six of the varieties examined represented higher yielding lines available in limited quantities from the *T. vogelii* breeding program at the Federal Experiment Station, Crops Research Division, ARS, USDA, Mayaguez, Puerto Rico. These were BL651, BL654, BL656, BL657, BL659, and BL6285. They were grown at Glenn Dale, Md., during the 1968 season. The seventh variety (PI257533) was a plant selection that was not part of this breeding program but of which larger quantities of seed were available. A few plants of PI257533 were grown in Peoria, Ill., in 1968 as part of this rotenoid distribution study, and larger plantings were made at Lafayette, Ind., in 1967 and 1968 for the fractionation studies. All plants were approximately 4 months old when harvested between September 30 and October 18.

Plant Processing. For the fractionation studies, the PI257533 *T. vogelii* from Lafayette was passed through a forage chopper ("Giant" model, Taylor, Stiles, and Co., Riegelsville, N.J.). The 1967 plants were chopped into $^1/_2$-in. lengths to facilitate passage through the feed screw on several of the mills. For the 1968 plants a 2-in. chop (see Figure 1D) was chosen as being better suited to the single machine with which they were further processed. The chopped material was then spread 1 in. thick on wire screens and dried in a large, forced-draft oven immediately before processing. For the study of rotenoid losses during drying, the material was heated in an oven at 120° C for 5, 10, 20, 40, or 80 min. For the effect of drying on milling, the chopped plants were oven-dried at 80° C for 40, 80, or 160 min, or at 120° C for 20, 40, or 80 min.

Chopped and dried plants were further processed in either of three different machines: a 6-in., center-feed hammer mill (Raymond Pulverizer Div., Combustion Engineering, Inc., Chicago, Ill.) operated without the usual screen; a disc attrition mill (Style 148, size 8, Bauer Brothers Co., Springfield, Ohio) with a pair of opposed discs with spiked teeth ($^6/_{16}$ by $^7/_{16}$ in. tapering to $^2/_{16}$ by $^7/_{16}$ in.) set at 0.1 in. clearance; or an air classifier (laboratory model, Raymond Pulverizer Div.) with a large fan and a single eight-bladed

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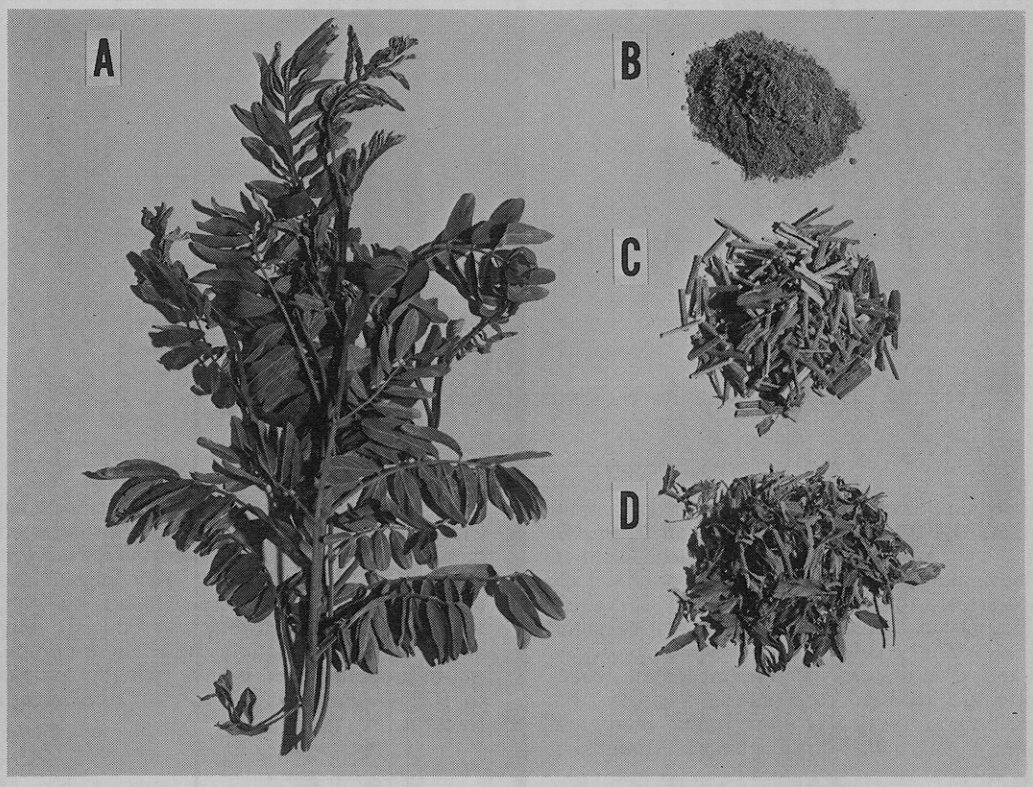

Figure 1. *Tephrosia vogelii*

A. Whole plant. B. Leaflet fraction from an air classifier. C. Stem-petiole fraction from an air classifier. D. Chopped and dried plant

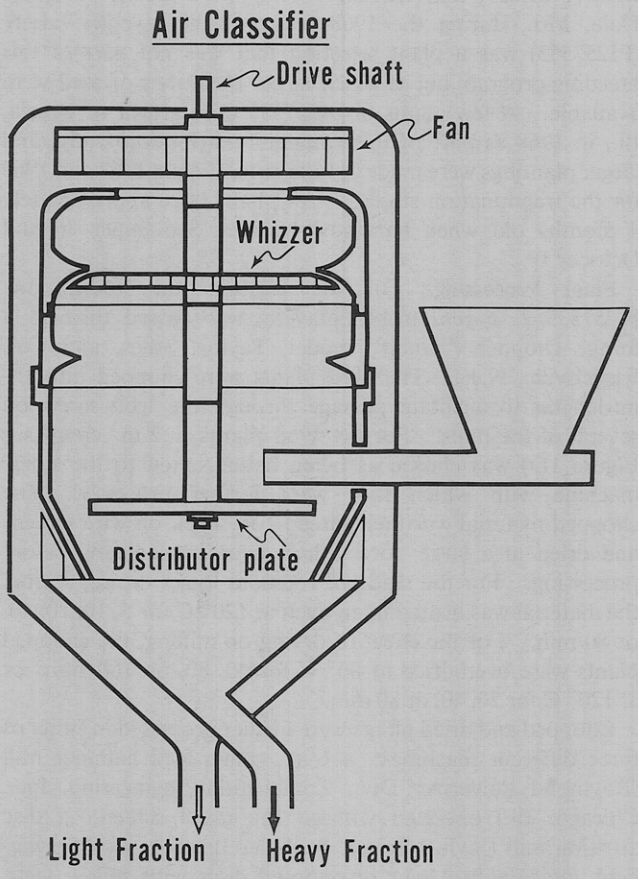

Figure 2. Cross-sectional view of air classifier used to fractionate chopped plants

See Experimental Section for details

"whizzer" in the upper position. The chopped and dried material enters the inner chamber (see Figure 2) through a funnel and falls to a rotating distributor plate which scatters the mixture into the rising airstream. Whizzer blades bat the tougher, heavier pieces down into a heavy-fraction collector, and mill the friable leaflets until fine enough to pass the blades. The leaflet particles then pass up into the outer chamber of the separator and hence down into a light-fraction collector. The air classifier thus separates the material into fine and coarse fractions without the use of sieves. Sieving of the output from the two mills is necessary, however, for partial separation of the somewhat finer leaflet from the coarser stem and petiole particles. The milled product was passed through a set of U.S. standard test sieves: 1/4 in. (0.250 in.), No. 8 (0.0937 in.), No. 12 (0.0661 in.), No. 20 (0.0331 in.), and No. 30 (0.0232 in.). Fractions were examined under a microscope to estimate their content of the various plant components. The intermediate fractions containing more than approximately one-third leaflet fragments were pooled with the other leaflet fractions, the rest, with the stem-petiole fractions. (Throughout this report the term "petiole" will include both petiole and rachis.) While this separation criterion is somewhat arbitrary, the size of the intermediate fractions was too small (20% of total) to alter any of the conclusions.

Analysis. Samples for analysis were taken from each of the fractions just described and from each of the component parts of the fresh plants included in this study. The fresh samples were composites of from four to nine individual plants. For extraction and further analysis, one sample was taken of the petioles and roots and two of the leaflets and stems of the Maryland and Illinois plants. Of the Indiana plants two samples of each fraction or treatment and six to

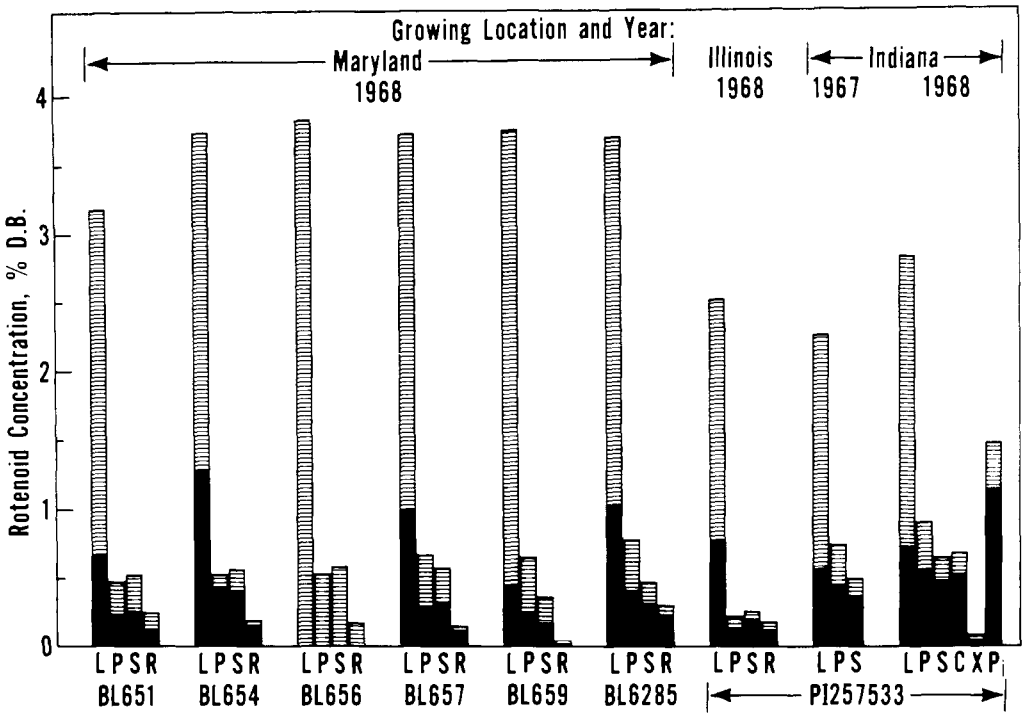

Figure 3. Concentration of rotenoids in various parts of seven varieties of *T. vogelii*

Abbreviations: L = leaflet; P = petiole (including the rachis); S = stem; R = root; C = stem cortex plus epidermis; X = stem xylem; Pi = stem pith. The height of the bar indicates the concentration of rotenone (solid portion) plus deguelin (hatched portion)

nine of the fresh plant parts were analyzed. Those samples not already pulverized were chopped or shredded to give pieces $^1/_4$ in. long, or less. They were then extracted with 10 to 15 ml of acetone per g dry matter for 48 hr at 25° C. The extracts were stored at −15° C until analyzed for rotenoids or chlorophyll. Moisture contents were determined by drying to constant weight at 120° C. All analytical results are expressed on a dry basis.

Rotenone and deguelin were determined by thin-layer densitometry (Delfel and Tallent, 1969) at least in duplicate. A double-beam densitometer (Model SD 3000, Schoeffel Instrument Co., Westwood, N.J.) was substituted for the single-beam instrument previously used. The monochromator of the new instrument was set at a wavelength of 540 nm. Chlorophyll was measured spectrophotometrically in suitably diluted aliquots of the rotenoid extracts from the plant fractionation study. Since the spectra of these aliquots matched the spectrum of chlorophyll *a* in acetone given by Mackinney (1940), his absorptivity value of 84.0 l. per g cm was used to calculate chlorophyll concentration from absorbance measurements at 661 nm.

Both rotenoid and chlorophyll concentrations were used to estimate the purity of the mechanically separated fractions. ("Purity" in this report means the freedom from contamination of leaflets by stems and petioles or *vice versa*.) First the concentration of rotenoids or chlorophyll was corrected for decrease during drying and milling by multiplying by the concentration in the fresh plant and dividing by the concentration in the processed plant (recombined leaflet plus stem-petiole fractions). This correction factor ranged from 1.16 to 1.72. The per cent purity in the leaflet fraction (P_L) or in the stem-petiole fraction (P_S) was then calculated as follows:

$$P_L = 100\,(L_F - S_O)/(L_O - S_O)$$

$$P_S = 100\,(L_O - S_F)/(L_O - S_O)$$

where:

L_F = corrected concentration of total rotenoids (rotenone plus deguelin) or of chlorophyll in the leaflet fraction.

S_F = the same for the stem-petiole fraction.

L_O = concentration of total rotenoids or of chlorophyll in the fresh (hand-separated) leaflets.

S_O = the same for the fresh stem plus petiole mixture.

RESULTS AND DISCUSSION

Rotenoid Distribution. In *T. vogelii*, the concentration of total rotenoids, indicated by the overall height of the bars in Figure 3, was far higher in leaflets than in petioles and stems, and was lowest of all in roots. This finding is in agreement with the data of Worsley (1939) and of Irvine and Freyre (1959). Worsley further found that the specialized rotenoid-storage cells tended to occur more frequently in certain tissues within an organ than in others. In the stem these cells were concentrated primarily in the pith and the cortex, with few such cells seen in the xylem. Worsley's results concur with those in Figure 3 for the stem of PI257533.

The individual levels of rotenone and deguelin within the plant roughly paralleled their sums (total rotenoids): the leaflet had the highest levels; the root, the lowest. Of particular interest is BL656; it contains no rotenone, yet has deguelin concentrations comparable to the total rotenoid concentrations of the other varieties. In each of the plants tested, the leaflets had more deguelin than rotenone; whereas the reverse was generally found in the petioles, stems, and roots. The ratio of rotenone to rotenone plus deguelin (Table I) showed an interesting general pattern. The order of the plant parts when ranked according to this ratio was: leaflet < petiole < stem < root (differences between plant parts were statistically significant, $\alpha = 0.05$). Within the stem of PI257533 the ratio was constant: cortex plus epidermis, 0.79; xylem, 0.80; and pith, 0.76.

Table I. Ratio of Rotenone to Rotenone Plus Deguelin in the Leaflet, Petiole, Stem, and Root of *T. vogelii* Varieties

Plant Part	Growing Location[a] and Year: Md. 1968	Md. 1968	Md. 1968	Md. 1968	Md. 1968	Md. 1968	Ill. 1968	Ind. 1967	Ind. 1968
	Variety: BL651	BL654	BL656	BL657	BL659	BL6285	PI257533	PI257533	PI257533
	Rotenoid Ratio								
Leaflet	0.21	0.34	0.00	0.27	0.12	0.28	0.30	0.25	0.26
Petiole	0.51	0.67	0.00	0.46	0.36	0.52	0.62	0.59	0.63
Stem	0.50	0.71	0.00	0.55	0.46	0.69	0.74	0.75	0.75
Root	0.50	0.79	0.00	0.79	0.50	0.76	0.75	...	...

[a] Md. = Glenn Dale, Md.; Ill. = Peoria, Ill.; Ind. = Lafayette, Ind.

Table II. Moisture Content of Leaflet and Stem-Petiole Fractions

Milling Technique	Fraction	Drying Time, Minutes: 20	40	80	160
		Moisture Content, %			
	Drying Temperature: 80° C				
Hammer mill	Leaflet	...	8	7	6
	Stem-petiole	...	8	7	6
Disc mill	Leaflet	...	10	8	7
	Stem-petiole	...	10	8	6
Air classifier	Leaflet	...	5	9	8
	Stem-petiole	...	11	9	8
	Drying Temperature: 120° C				
Hammer mill	Leaflet	7	6	8	...
	Stem-petiole	10	6	5	...
Disc mill	Leaflet	11	7	6	...
	Stem-petiole	11	7	5	...
Air classifier	Leaflet	14	7	4	...
	Stem-petiole	29	10	2	...
Air classifier[a]	Leaflet	13	7	4	...
	Stem-petiole	59	45	13	...

[a] 1968 plants chopped 2 in. long; all others 1967 plants chopped ½ in. long.

Table III. Relationship of Drying Time in an Oven at 120° C to Moisture Loss and to Rotenoid Recovery in Whole, Chopped *T. vogelii* Plants[a]

Drying Time, Minutes	Moisture Content, %	Rotenoid Recovery, %: Rotenone	Deguelin
0	72	100	100
5	61	97	102
10	58	87	83
20	35	82	83
40	23	88	80
80	7	63	69

[a] Variety PI257533 chopped 2 in. long.

Barnes *et al.* (1967) planted four *T. vogelii* varieties in three locations and found differences of from 10 to 34% in rotenoid levels in the leaves (*i.e.*, leaflets plus petioles) due to growing location. We found a similar range (22%) in the leaflets of variety PI257533 due to location and season, but much larger ranges (124 and 90%, respectively) in the petioles and stems. The ratios of rotenone to rotenone plus deguelin for this variety were generally more constant (Table I). Our limited data suggest that absolute rotenoid levels in *T. vogelii* are more influenced by environment than are rotenoid ratios. Apparently, the ratios are more characteristic of a given variety.

Fractionation Studies. Low rotenoid levels in petioles and stems shown in the distribution study make it highly desirable for these materials to be removed before the leaflets are extracted commercially. Variety PI257533 was chosen for the leaflet *vs.* stem-petiole separation trials because sufficient seed was available for growing the quantities of plant material required. The distributions of dry matter within the plants grown in 1967 and 1968, respectively, were: leaflet, 46.3 and 52.2%; petiole, 9.4 and 10.4%; and stem, 44.3 and 37.4% of the whole plant.

Preliminary drying was necessary before milling. Moisture contents of the separated fractions are listed in Table II. In the hammer and disc mills, the entire plant had to be quite dry to avoid caking the inside of the machines. The drying required was facilitated when the plants were chopped into short lengths. For efficient milling in the air classifier, only the leaflets needed to be dry (preferably around 15% moisture). In one trial with the leaflet moisture about 41%, the throughput was reduced and the machine eventually clogged. On the other hand, stem moisture was not critical since the stem was rejected intact. See, for example, the high moisture contents in the stem-petiole fraction at the bottom of Table II. Consequently a longer chop can be used with the air classifier, and the completeness of the separation is thereby increased.

Oven drying the chopped fresh plant at 120° C for extended periods reduced the recovery of rotenoids (Table III). Barnes and Freyre (1966) have reported larger losses when dried samples were reheated. This result indicates that evaporation of moisture from the fresh plant may slow the temperature rise within the plant material and hence afford some protection to the rotenoids. In this study the rates of destruction of rotenone and deguelin did not significantly differ from each other, in spite of their different distributions within the plant. Since deguelin is concentrated primarily in the leaflets, and since the leaflets dry more rapidly than other parts of the plant, different rates of destruction of rotenone and deguelin might have been anticipated. Although the magnitude of the losses will probably vary depending on the temperature, design, and loading of the oven, obviously it is best to restrict plant drying to the minimum required by subsequent milling and extraction processes.

In addition to the decrease of rotenoids in the drying step, another problem became apparent during the fractionation studies that was serious with two of the procedures tested. During milling, a loss of rotenoids occurred, possibly due to frictional heating of the finely ground plant particles. The average loss from processing (excluding drying) for the hammer and disc mills was 18 and 24%, respectively, and for the air classifier, 6%. There was some indication that losses from this source might depend on sample dryness. For

Table IV. Total Rotenoid Concentrations in Leaflet Fractions of *T. vogelli*

Milling Technique	Drying Time, Minutes: 20	40	80	160
	Rotenoid Concentration,[a] % Dry Basis			
Drying Temperature: 80° C				
Hammer mill	...	1.05	1.23	1.38
Disc mill	...	1.02	1.05	1.18
Air classifier	...	1.34	1.65	1.60
Drying Temperature: 120° C				
Hammer mill	1.21	1.40	1.20	...
Disc mill	1.13	1.07	1.06	...
Air classifier	1.75	1.53	1.39	...
Air classifier[b]	2.31	1.80	1.73	...

[a] Rotenoid levels in leaflets of fresh, unprocessed variety PI257533: 1967 plants, 2.24%; 1968 plants, 2.82%. [b] 1968 plants; all others 1967 plants.

Table V. Effect of Drying Temperature and Milling Technique on the Purity of *T. vogelii* Fractions

Milling Technique	Leaflet Drying Time, Minutes: 20	40	80	160	Stem-Petiole Drying Time, Minutes: 20	40	80	160
	Purity,[a] %				Purity,[a,b] %			
Drying Temperature: 80° C								
Hammer mill	...	61	66	78	...	72	85	98
Disc mill	...	64	65	76	...	77	81	93
Air classifier	...	77	87	79	...	96	106	117
Drying Temperature: 120° C								
Hammer mill	66	87	88	...	82	92	97	...
Disc mill	74	66	76	...	73	76	96	...
Air classifier	89	73	75	...	111	107	110	...
Air classifier[c]	97	90	95	...	92	96	109	...

[a] Calculated from the total rotenoid concentration as under Experimental. [b] Values higher than 100% result from the loss of rotenoid-rich epidermis and cortex from stem-petiole to the leaflet fraction. [c] 1968 plants (PI257533) chopped 2 in. long; all others 1967 plants chopped 1/2 in. long.

example, air classification of material oven-dried 20 min at 120° C gave no additional losses of rotenoids.

The major objective of the fractionation studies was to maximize rotenoid concentration in the recovered leaflet fraction and thus to minimize extraction costs. Table IV shows that the air classifier was clearly better in this respect than either of the other two fractionating techniques. The best combination of variables studied was air-classification of material oven-dried at 120° C for 20 min. This combination gave a rotenoid concentration 78% as high as that of the fresh leaflets with the 1967 plants, and 82% as high with the 1968 plants. Corresponding leaflet recovery values were 123 and 107%, respectively (higher than 100% due to contamination by the stem-petiole fractions). Physical losses during processing were 3% or less. Application of the best processing conditions to the highest rotenoid leaflets in Figure 3 would have given essentially quantitative recovery of a leaflet fraction having more than 3% total rotenoids.

The concentrations in Table IV are lower than in the fresh leaflet because of three factors: losses during drying, losses during subsequent processing, and contamination by the low-rotenoid stems and petioles. The magnitude of the first two factors has already been discussed. The purity calculation given in the Experimental section was used in determining the magnitude of the cross-contamination. This calculation is based on the fact that both rotenoids and chlorophyll are concentrated more in leaflets than in stems and petioles. Hence, if a fraction recovered has the same concentration of the component (after correction for processing losses) as the fresh leaflet or stem plus petiole, it would be 100% pure. If its concentration were halfway between the two, the purity would be 50%.

Effects of drying and of the milling and fractionating technique on purity of the fractions produced were studied first on the basis of total rotenoid data. With the hammer and disc mills, the completeness of separation of both the leaflet fraction and the stem-petiole fraction tended to increase with increasing drying time (Table V). In contrast with these two mills, the air classifier gave cleaner fractions and, moreover, gave the best separations with the shorter, and therefore more desirable, drying times. As anticipated, the air classifier also gave better results with *T. vogelii* chopped 2 in. long (Table V, bottom row) rather than 1/2 in. long since the larger stem and petiole pieces were easier to reject. Samples of the fractions produced by the air classifier with the 1968 plants dried 20 min at 120° C may be seen in Figures 1B and C.

Estimation of fractionation efficiency from the chlorophyll concentration provided an independent check on the conclusions based on rotenoid data. Fraction purity results based on chlorophyll data closely agree with those based on rotenoid data (Figure 4). Although the chlorophyll estimates tended to be slightly lower, both sets of data gave essentially the same results. Chlorophyll measurement might provide a convenient and rapid means of following a commercial fractionation patterned after studies reported here.

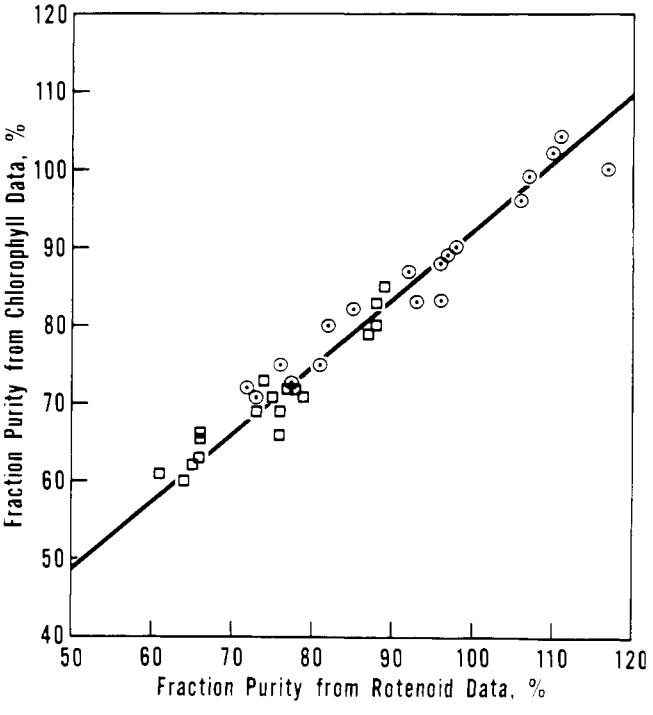

Figure 4. Correlation between purity estimates based on chlorophyll data and those based on rotenoid data for leaflet and stem-petiole fractions from *T. vogelii*

Method of purity calculation is explained under Experimental. □, leaflet data; ⊙, stem-petiole data

The efficiency of the separation process is related to two factors: (1) the purity of the leaflet fraction, and (2) the purity of the stem-petiole fraction. The first is important in providing the highest quality product for extraction; the second, in minimizing rotenoid losses to the discard. From either standpoint the most efficient process, based either on rotenoid or on chlorophyll results, was air classification.

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