ISOLATION AND CHARACTERIZATION OF THE INSECTICIDAL FRACTION FROM LEAF EXTRACTS OF *Tithonia diversifolia* A. Gray

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ABSTRACT

The different fractions of the acetone extract of *Tithonia diversifolia* A. Gray leaves were separated by preparative layer chromatography after precipitation of pigments and more polar constituents with lead acetate. The insecticidal fraction was identified through insect bio-assay using *Dysdercus cingulatus* Fab., *Tribolium castaneum* Herbsr. and *Sitophilus zeamais* Motsch. The median lethal dose (LD50) of the active fraction was obtained for these species and for *Plutella xylostella* L. and *Spodoptera exempta* Walker. The toxicity of the semi-purified isolate was only 24.47% less toxic (LD50 = 1.3657 mg/g) than malathion (LD50 = 1.0972 mg/g) to *P. xylostella,* but was much less toxic than the said insecticide to the other test species. The toxicity of the isolate to *P. xylostella* was comparable with values obtained for synthetic insecticides used for this insect. Characterization of the active fraction suggested the presence of a gamma-lactone with a hydroxyl group attached to the ring or to an alkyl substituent. Unsaturation may be present in the ring itself, or external to the ring, alpha to the carbonyl.

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KEY WORDS: *Tithonia diversifolia.* Botanical insecticide. Isolation Preparative layer chromatography. Toxicity. Median lethal dose. Gas chromatogram. Infrared spectra. Gamma lactone.

INTRODUCTION

The continuous use of synthetic insecticides has given rise to several ecological problems such as environmental contamination by residues, health hazards, undesirable effects on non-target organisms and the rapid development of insect biotypes resistant to the action of many synthetics. With the increasing awareness of such adverse

effects, interest on naturally occurring plant products with insecticidal activity is inevitable.

The revival of interest in biologically active chemicals of nature is motivated by two long-range objectives: to identify the possible sources of new and effective botanical insecticides and to elucidate the chemical structure of the active components. Botanical insecticides extracted on a large scale may be used to replace or at least supplement the activity of existing synthetics against resistant insects. Meanwhile, structural elucidation of the active components may lead to valuable insights on structure-activity relationships. Furthermore, plant metabolites may serve as design models for the chemical synthesis of new compounds with improved activity and more desirable properties as in the case of pyrethroids (Mrak, 1973).

Researches on plants with insecticidal components are presently receiving considerable attention in other countries. In the Philippines, however, the search for novel botanicals seems to lag behind, with relatively few studies being conducted on local plant species. Fewer still are studies conducted to elucidate the chemical structure or at least characterize the insect-active components of local plants.

A preliminary screening was performed on extracts of roots, flowers and leaves of nine Compositae species. *Tithonia diversifolia* A. Gray leaf extracts were identified as the most promising ones assayed, exhibiting high toxicity to three species of insects. This paper presents 1) the method used to isolate the insecticidal fractions from *T. diversifolia* leaves, 2) the activity of the isolated fraction against five species of insects, and 3) the characterization of the active isolate through physical and chemical properties which may be indicative of the chemical structure of their components.

MATERIALS AND METHODS

Extraction of **T. diversifolia** *Leaves.* — Fully mature but non-senescent leaves of *T. diversifolia* were separated from the rest of the collected plant materials. Leaves were oven-dried at 40-45°C for 48 hr prior to grinding in a Wiley mill with a 20-mesh screen.

Extraction was performed by overnight soaking of 1 kg of ground leaves in 2.5 li of acetone. The extracts were then separated from the solids by filtration using suction. Extraction of the same plant material was performed twice, combining the filtrate from each extraction. The combined filtrates were then concentrated *in vacuo* using a rotary evaporator.

Isolation of the Active Fraction from the Extract. — The crude leaf extract concentrate was taken up in 1 li of ethanol warmed to 60°C. The resultant dark green solution was then mixed with 1 li of aqueous lead acetate solution (4%). After agitation, the cloudy mixture was filtered through celite to obtain a clear, light yellow filtrate.

The filtrate was concentrated *in vacuo* to remove the ethanol added, and the organic components present in the filtrate were extracted into chloroform. The extract was then dried with anhydrous magnesium sulfate, filtered and evaporated *in vacuo* to a viscous golden syrup.

Isolation of the components of the clarified extract was performed using preparative layer chromatography with silica gel as adsorbent. Plates were spotted with the extract and were developed in 1:1 chloroform-diethylether. Spots were temporarily visualized using iodine crystals. Spots with similar Rf values were scraped off from the plates, and isolates were recovered from the adsorbent with chloroform. Chloroform was then completely removed by evaporating *in vacuo*.

Toxicity Tests.— The test insects used to identify the active fractions were 6- to 8-day-old cotton stainer nymphs *(Dysdercus cingulatus* Fab.), 2- to 3-week-old adults of the red flour beetle *(Tribolium castaneum* Herbst.) and similarly aged corn weevil *(Sitophilus zeamais* Motsch.). The toxicity of the active fraction thus identified was evaluated on the three species mentioned, on 6- to 7-day-old larvae of the diamond-back moth *(Plutella xylostella* L.) and on 8-day-old larvae of the black armyworm *(Spodoptera exempta* Walker). *D. cingulatus* nymphs were inactivated with carbon dioxide gas or low temperature (10°C) prior to application of the test solutions.

Test solutions were topically applied on the notum of *D. cingulatus, P. xylostella,* and *S. exempta,* and on the mid-abdominal sternites of *T. castaneum* and *S. zeamais.* One µl of the test solutions was applied on each insect, using a Burkard microapplicator with a calibrated syringe to deliver the volume required.

Sixty insects from each test species were used for each solution. In batches of twenty, treated insects were kept in petri dishes lined with white paper discs for ease in data gathering. Food provisions were not necessary for treated *T. castaneum* and *S. zeamais,* while all the other test species needed food for their sustenance during the 48-hr observation. Corrected percentage mortality was calculated using the formula of Abbott (1925).

The median lethal dose (LD50) of the active fraction on the test insects was determined from the results of topical application of prepared solutions at graded concentrations. Calculations for LD50 values were through probit analysis (Finney, 1952) in µg/insect, but values obtained were subsequently converted to mg/g body weight for comparison of susceptibilities of the species tested.

Characterization of the Active Fraction.— The active fraction was characterized based on its physical and chemical properties. The solubility of the fraction in different solvents (i.e.: water, dilute acid, dilute alkali, cold concentrated sulfuric acid, acetone, chloroform, diethylether, methanol, ethanol,

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hexane, petroleum ether and benzene) and its Rf value in 1:1 chloroform-diethylether were obtained.

A gas chromatogram and infrared (ir) spectrum of the samples were obtained from the International Rice Research Institute. The gas chromatogram was run on a Varian Gas-Liquid Chromatograph at the following specifications:

Column: 75 cm x 22 nm internal diameter; stainless steel; packed with 5% Carbowax 6000 on chromosorb W AW 100/200; oven temperature 80-180°C at 5 /min flow rate.
Carrier gas: nitrogen at 20 cc/min
Detector: flame ionization detector at 250°C
Attenuation: 32; at a range of 10^{-11} amp/mv
Flow rate: 1 cm/min

The ir spectrum was obtained by running the sample neat in a Perkin-Elmer Infrared Spectrometer.

To augment the information given by the ir spectrum, an elemental analysis of the fraction was performed, and classification tests for functional groups were carried out. The methods followed in the elemental analysis and classification tests were those of Shriner and his colleagues (1964).

RESULTS AND DISCUSSION

Isolation of the Active Fraction.

Precipitation of the pigments and the more polar plant metabolites with lead acetate and reextraction of the remaining components with chloroform gave a clear light-yellow solution. A viscous golden syrup was obtained upon the removal of chloroform *in vacuo*. From 1 kg of dried leaves for extraction, about 3.8 g of clarified extract concentrate was obtained.

Topical application of the thick syrup at a set dosage of 50 µg/insect gave 50%, 33% and 28% corrected mortalities for *D. cingulatus, T. castaneum* and *S. zeamais,* respectively. This indicated that the insecticidal constituents of the extract were not removed by lead acetate treatments.

Seven isolates were obtained through preparative layer chromatography (Fig. 1). Table 1 gives the relative location of the isolates in the chromatogram through their Rf values. The table also presents the weights of each isolate recovered from the adsorbents, and the percentages of recovery for each fraction.

Fig. 1 shows that the first three fractions (A, B and C) are relatively pure, while the remaining fractions are still mixtures of two or more components. Such is suggested by the sizes and shapes of the spots obtained in the chromatogram.

Toxicity of Fractions.

Among the insects tested, *D. cingulatus* nymphs were most sensitive to the action of the isolated fractions (Table 2). The said species was susceptible to the action of four

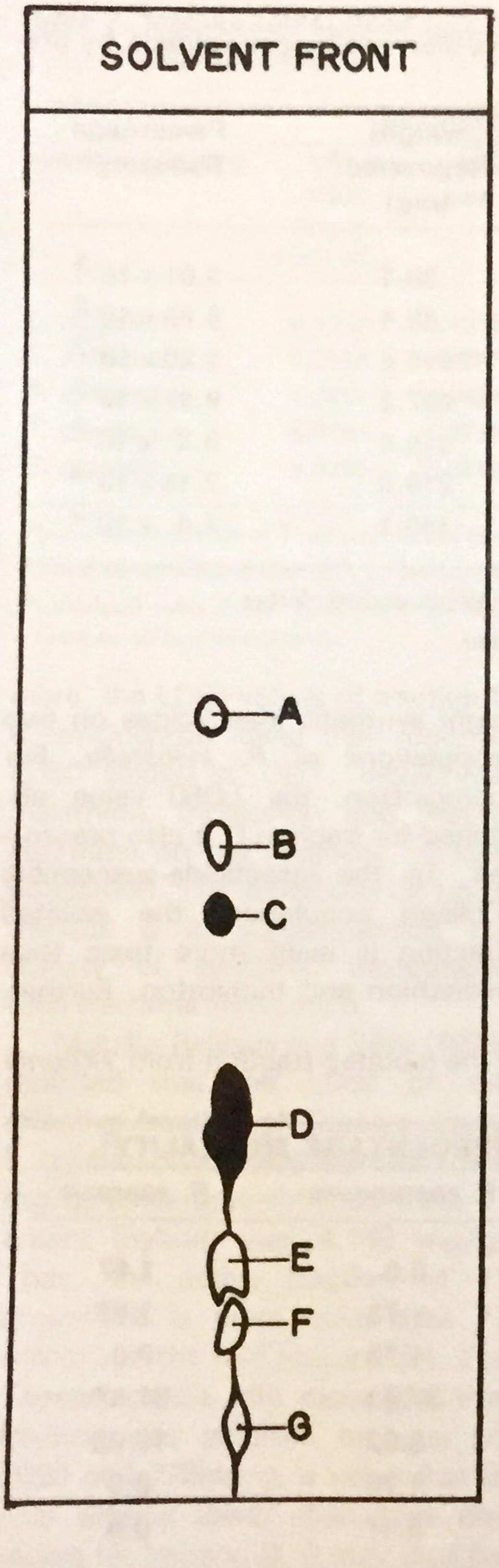

Fig. 1. Thin layer chromatogram of *T. diversifolia* leaf extract in 1:1 chloroform-diethylether.

of the isolates (A, B, C and D). The other two test species were susceptible only to the action of fraction D(Rf = 0.26). As a consequence, fraction D was selected for subsequent investigations.

Fraction D. Fraction D was most toxic to *D. cingulatus* nymphs, as indicated by the low LD50 value obtained on this insect species (Table 3).

In the course of obtaining mortality data, it was also observed that ***D. cingulatus* nymphs tended to molt** prematurely when treated with fraction D at dosage below 0.5 µg/insect. Such observation was based on the number of exuviae found on the petri dishes containing the treated insects. This premature molting deserves further investigation, since this may be indicative of the presence of a growth regulator in fraction D. Inasmuch as premature molting was not observed in *P. xylostella* and *S. exempta* larvae, this probable growth regulator may be specific for *D. cingulatus.*

Fraction D is more toxic to the soft-bodied test species than to those with highly sclerotized exoskeleton. Such differences in toxicity could probably be explained by the more efficient penetration barrier afforded by the highly sclerotized integument of *T. castaneum* and *S. zeamais.*

Fraction D was only 24.47% less toxic than malathion to *P. xylostella* although it was very much less toxic to the other test insects. The comparable toxicity of this fraction to malathion is important in view of the reports of Barroga and Morallo-

Table 1. Fractions isolated from *Tithonia diversifolia* leaf extracts by preparative layer chromatography.

Fraction	Rf Value [1]	Weight Recovered [2] (mg)	Percentage Recovery [2]
A	0.55	30.1	3.01×10^{-3}
B	0.47	88.6	8.86×10^{-3}
C	0.37	219.8	2.20×10^{-2}
D	0.26	997.2	9.97×10^{-2}
E	0.17	318.4	3.2×10^{-2}
F	0.12	216.0	2.15×10^{-2}
G	0.05	140.1	1.4×10^{-2}

[1] Solvent system used for development was 1:1 chloroformdiethylether.

[2] Based on 1 kg of plant material used for extraction.

Rejesus (1976; 1981) on the developing resistance of *P. xylostella* population to DDT, dichlorvos, malathion, trichlorfon and methyl parathion. The said insecticides are commonly used in areas where *P. xylostella* populations are high.

Table 4 shows the toxicity of eight synthetic insecticides on two populations of *P. xylostella*. For comparison, the LD50 value obtained for fraction D is also presented. To the insecticide-susceptible College population, the isolated fraction is even more toxic than malathion and trichlorfon. Further-

Table 2. Insect toxicity performance of the isolated fraction from *Tithonia diversifolia* leaf extract.[1]

Fraction	CORRECTED PERCENTAGE MORTALITY[2]		
	D. cingulatus	*T. castaneum*	*S. zeamais*
A	36.67	0.0	1.67
B	50.00	1.73	1.67
C	26.67	1.73	0.0
D	60.00	37.93	31.67
E	23.33	8.62	10.00
F	6.67	1.73	0.0
G	13.33	5.17	0.0

[1] Evaluated at a set dosage of 10 µg/insect.

[2] Average of three replicates with 20 insects per replicate. Mortality observations obtained 48 hr after treatment; corrected using Abbot's formula.

Table 3. Median lethal dose (LD50) of fraction D[1] and malathion on five insect species.

INSECT SPECIES	FRACTION D			MALATHION[2]		
	Confidence Limits (95%)			Confidence Limits (95%)		
	LD50C (mg/g body wt.)	Lower	Upper	LD50 (mg/g body wt.)	Lower	Upper
D. cingulatus	0.1136	0.1031	0.1241	0.0007	0.0006	0.0008
S. exempta	1.2858	1.2787	1.2930	0.0070	0.0040	0.0100
P. xylostella	1.3657	1.3421	1.3889	1.0972	0.9861	1.2361
T. castaneum	5.0784	5.0716	5.0851	0.0810	0.0619	0.0952
S. zeamais	5.8296	5.8242	5.8350	0.0070	0.0060	0.0080

[1] Insect-active fraction of *T. diversifolia* leaf extract.

[2] Based on unpublished data of P. A. Javier, 1981.

[3] Calculated using Probit Analysis, based on 60 insects per dosage applied; mortality obtained 48 hr after treatment.

more, the LD50 values of fraction D is considerably lower than reported LD50 values of DDT, dichlorvos, malathion, trichlorfon and methyl parathion on the resistant Trinidad population. The possibility of using fraction D in supplementing the activity of synthetic insecticides then warrants verification.

Morallo-Rejesus and Silva (1979) reported that the LD50 of the effective fraction of *Tagetes erecta* L. (native) on *P. xylostella* was 1.684 mg/g, while that obtained from *T. erecta* (hybrid) was 4.149 mg/g. Thus, the active fraction of *T. diversifolia* is more toxic than *T. erecta's* to the said test species. For *Tagetes patula,* the most effective fraction was reported to have an LD50 of 1.228mg/g, a value that is only slightly lower than that observed for fraction D. It may thus be said that at the very least, the active constituents of *T. diversifolia* leaves are comparable to those of *T. erecta* and *T. patula.*

Characterization of the Active Fraction.

Fraction D, the active fraction of *T. diversifolia* leaf extract, had an Rf value of 0.26 on silica gel g-coated plates developed in 1:1 chloroform-diethylether. The fraction, upon recovery from the adsorbent, appeared as a very viscous golden yellow oil.

The oil was completely miscible with acetone, diethylether, chloroform, ethanol and methanol. The solubility of fraction D in hexane, petroleum ether and benzene, however, was relatively lower than that observed for the former organic solvents mentioned.

Fraction D was insoluble in water, dilute hydrochloric acid and dilute sodium bicarbonate. It was slightly soluble in 5% sodium hydroxide. The oil was also soluble,

Table 4. Median lethal doses (mg/g) of different insecticides on College and Trinidad populations of *P. xylostella* as obtained by different researchers.

Researchers	Insecticide	College[1]	Trinidad[2]
Barroga and Morallo-Rejesus (1981)	Mevinphos	**0.04**	**0.04**
Barroga and Morallo-Rejesus (1976)	Diazinon	**0.0634**	**0.594**
Barroga and Morallo-Rejesus (1981)	EPN	**0.07**	**0.22**
Barroga and Morallo-Rejesus (1976)	DDT	**0.224**	**2.414**
Barroga and Morallo-Rejesus (1976)	Dichlorvos	**0.338**	**2.50**
Javier (1981) and Barroga and Morallo-Rejesus (1981)	Malathion	**1.0972**	**16.91**
Barroga and Morallo-Rejesus (1981)	Trichlorfon	**3.13**	**18.20**
Barroga and Morallo-Rejesus (1976)	Methyl parathion	**5.689**	**31.966**
	Fraction D[3]	**1.3657**	

[1] Considered as insecticide-susceptible population.

[2] Considered as insecticide-resistant population.

[3] Insecticidal fraction isolated from *T. diversifolia* leaves. Median lethal dose taken from this study.

with decomposition, in cold, concentrated sulfuric acid.

The boiling point of the viscous oil was not obtained because of the rapid discoloration of the sample upon heating above 80°C. At reduced pressure (122 mm mercury), the oil failed to boil at 78°C.

The gas chromatogram of the fraction showed seven peaks (Fig. 2), with the second peak as the major component. The number of peaks could be indicative of the number of components present but the rapid discoloration observed upon heating above 80°C suggests the decomposition of the components at elevated temperatures. An observed peak in the gas chromatogram could correspond to either a component of the isolated fraction or to degradation products of a component of the fraction.

Elemental analysis of the oil through sodium fusion showed that only carbon, hydrogen and oxygen

were present. The sample was also observed to burn with a yellow non-sooty flame.

The ir spectrum of fraction D is shown in Fig. 3. The absence of strong absorptions in the 909 to 650 cm^{-1} region indicates a non-aromatic structure, since aromatic and heteroaromatic rings display strong out-of-plane C-H bends and ring bend absorptions in this region (Silverstein and Bassler, 1967).

The strong, broad absorption band at 3460 cm^{-1} is indicative of an N-H or an O-H stretch. The absence of nitrogen, as determined by elemental analysis, strongly supports the assignment of this strong band to the O-H stretching frequency. The relatively low frequency of absorption suggests intermolecular hydrogen bonding. The greenish-blue opaque solution rapidly formed by the oil with chromic anhydride also supports this assignment, as well as indicating a primary or a secondary alcohol functionality (Shriner *et al.*, 1964).

The strong absorption at 1780-1740 cm^{-1} indicates the presence of a carbonyl or a carboxyl group. The carboxyl moiety, however, is unlikely since fraction D is only slightly soluble in dilute sodium hydroxide and is insoluble in dilute sodium bicarbonate. Furthermore, the ir spectrum fails to show the characteristic carboxyl absorption at the 3000-2500 cm^{-1} region (Shriner *et al.*, 1964). The absorption at 1780-1740 cm^{-1} is thus attributed to the carbonyl group of a ketone, an aldehyde, or an ester.

The presence of an aldehyde or a ketone moiety, however, is negated by the failure of fraction D to form dinitrophenylhydrazones with 2, 4-dinitrophenylhydrazine in concentrated sulfuric acid. In hydroxylamine hydrochloride, fraction D produced a distinct wine-red solution upon treatment with 5% ferric chloride, indicating an ester moiety. The absorption at 1780-1740 cm^{-1} was thus assigned to the C = 0 stretch of the carbonyl on an ester. This is supported by the presence of multiple absorption bands in the 1300-1000 cm^{-1} region, characteristic of the asymmetric C-O stretch (Silverstein and Bassler, 1967).

The relatively high carbonyl frequency observed suggests a lactone probably a γ - lactone, which characteristically absorbs at frequencies between 1795 and 1760 cm^{-1} (Silverstein and Bassler, 1967). The shift of absorption bands to slightly lower frequencies is suggestive of conjugation with a C = C bond.

Fraction D was able to discolor a 2% potassium permanganate solution rapidly, but it failed to decolorize bromine in carbon tetrachloride. This behavior may indicate unsaturation, if one considers that not all olefinic compounds take up bromine. The presence of a negative group on the carbon atoms of an olefinic linkage may cause slow addition of bromine or may even inhibit the addition reaction (Shriner *et al.* 1964). Furthermore, the strong ir absorption at 1670 cm^{-1} indicates an olefin, probably *trans*, di-, tri-, or even tetraalkyl-substi-

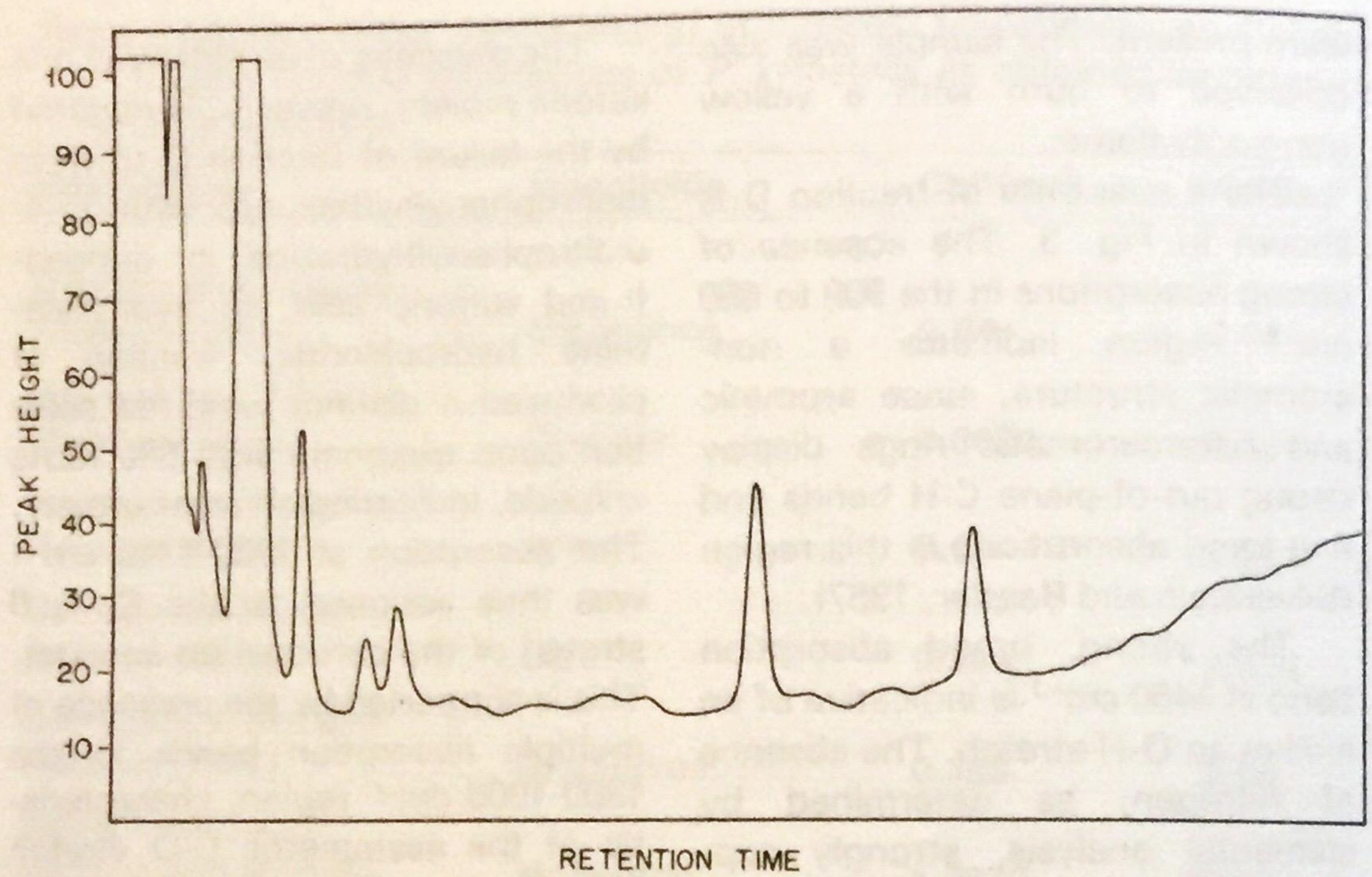

Fig. 2. Gas chromatogram of the active fraction from *Tithonia diversifolia* leaf extract.

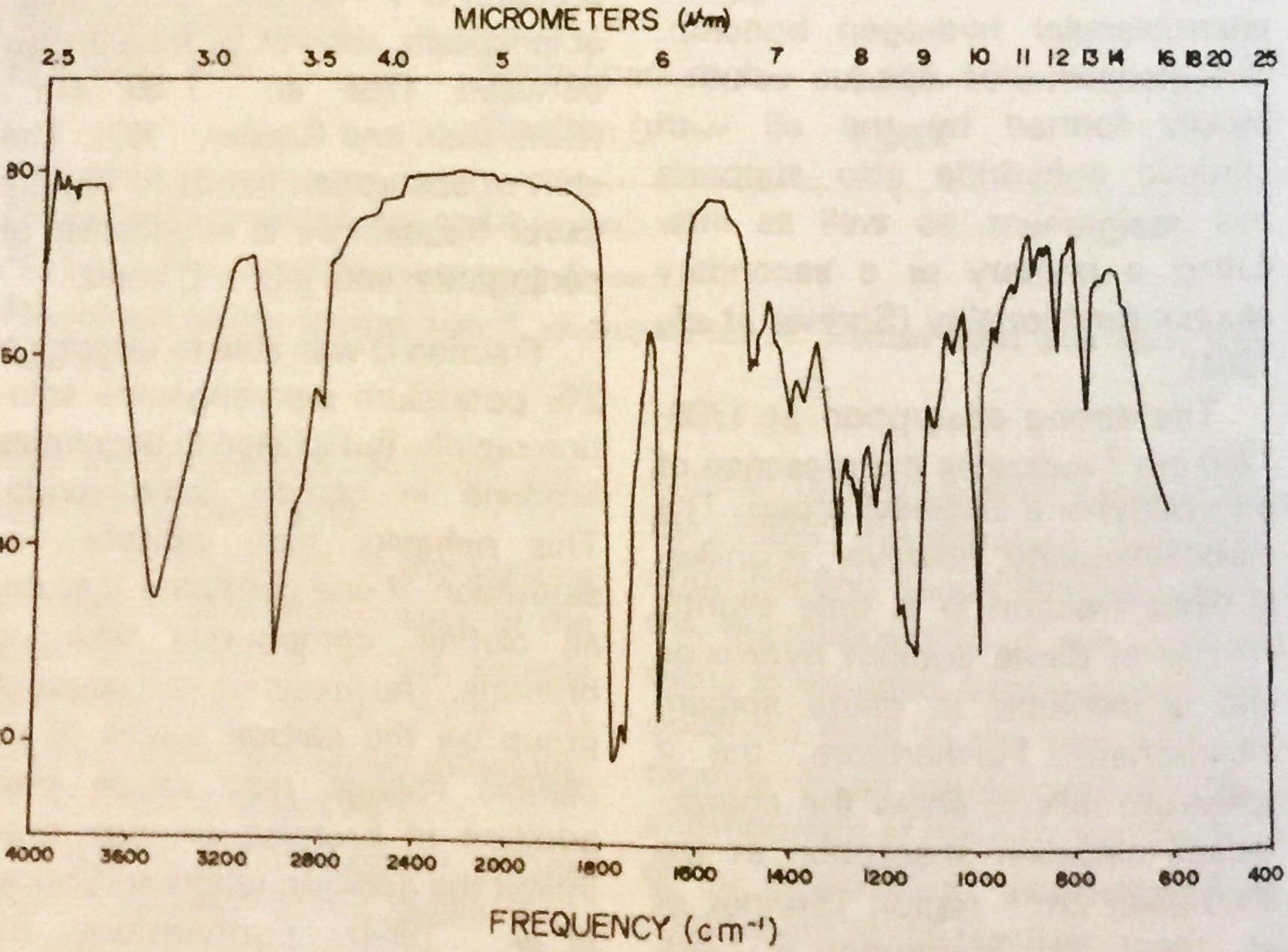

Fig. 3. Infra-red spectrum of the active fraction from *Tithonia diversifolia* leaf extract.

tuted (Silverstein and Bassler, 1967). The unsaturation, therefore, is probably ∝ to the carbonyl of the ɣ lactone.

Strong ir absorptions at 3000-2900 cm^{-1} were assigned to the C- H stretch of alkyl groups probably present as substituents on the ɣ -lactone ring (Silverstein and Bassler, 1967).

The active fraction thus appears to contain a ɣ -lactone with a hydroxyl group attached either to the lactone ring or to the alkyl substituent. The unsaturation may be present in the ring itself, ∝ to the carbonyl, as indicated by the strong ir absorption at 1670 cm^{-1} and by the failure of the sample to decolorize bromine in carbon tetrachloride.

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