Possible Confusion of Pyrethrins with Thiophenes in *Tagetes* species

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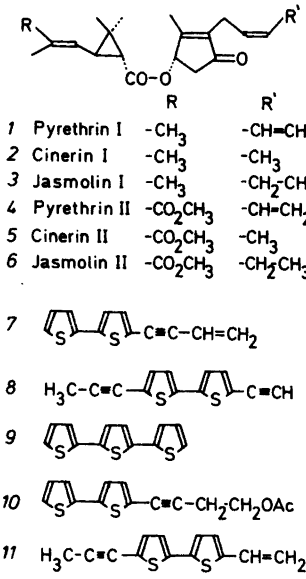
The pyrethrins, which comprise a group of six closely related monoterpenoid esters (*1*–*6*), are naturally occurring insecticides of considerable economic interest.[1] They were originally isolated from *Chrysanthemum cinerariaefolium* Vis. (formerly *Pyrethrum*), but have later been reported to be present also in *C. coccineum* Wild.,[1] *Achillea ageratum* L.,[2] *Tagetes erecta* L.[3] and *T. minuta* L.,[4] All of these plants belong to the *Asteraceae* (*Compositae*) family.

From a phytochemical point of view this apparent similarity in secondary metabolism between the genera *Chrysanthemum, Achillea* and *Tagetes* is somewhat surprising. Botanically it is common to divide the *Asteraceae* into thirteen tribes. *Chrysanthemum* and *Achillea* both belong to the 7th tribe, *Anthemideae,* while *Tagetes* belongs to the 6th, *Helenieae.* The secondary metabolites produced by plants in the two tribes are significantly different. In *Chrysanthemum* species polyacetylenes with spiro structures are dominant,[5] while the characteristic secondary metabolites of *Tagetes* species are thiophenes[6] (*7*–*10*). Indeed, the particular chemistry together with other characteristics has led to separation of *Tagetes* together with some other species into a new tribe, *Tageteae.*[7]

Previous work with callus cultures of *C. cinerariaefolium* has shown that pyrethrins were produced in a maximum total amount of 0.1 % of dry weight.[8] Also *Tagetes* species calli have been claimed to produce pyrethrins.[3,4]

We have undertaken studies of pyrethrin production of tissue cultures of pyrethrin producing species. Standards for our analyses have been commercially available extracts (25 % pyrethrins in mineral oil). A capillary GLC chromatogram of this extract freed from mineral oil is shown in Fig. 1a.[9,10]

In this case the mixture contained equimolecular amounts of pyrethrins. The low intensities of the less volatile compounds are particulary noticeable. The six pyrethrins were identified by GLC–MS analysis and by separation on column,



followed by 400 MHz ^{1}H NMR spectroscopy. The extraction, workup and GLC analysis procedures were tested on fresh flowers of *C. coccineum.* Two fluorescent bands were collected from preparative TLC plates. A mixture of extracts of these bands was shown by GLC (Figure 1b) to contain the six pyrethrins in varying amounts with a dominance of *4*. Good quantitative measurements were obtained by use of internal fatty acid methyl ester standards and the results (% dry weight of flowers) were: *1*(0.018), *2*(0.0007), *3*(0.001), *4*(0.15), *5*(0.001), *6*(0.02).

Various parts and calli of *T. erecta* L. and *T. patula* L. were treated in the same manner as *C. coccineum.* Two similar fluorescent bands were collected from TLC plates and analysed by GLC. Fig. 1c shows an analysis of the whole plant of *T. erecta.* None of the peaks in the chromatogram coincides with peaks in Fig. 1a or 1b. Conclusive evidence for non-identity was found in the GLC–MS analysis. The mass spectra of all the relevant compounds showed prominent M+2 peaks which strongly indicate the presence of one or more sulfur atoms. Moreover, in order to increase the sensitivity of the analysis, selected ion monitoring (SIM) was performed. Since the mass spectra of all six pyrethrins were recorded, characteristic peaks were known, including the molecular ion peaks. In the SIM analysis the computer searches for characteristic pyrethrin peaks in all the mass spectra of the *Tagetes* compounds. The characteristic *m/z* values employed in this case were: *1*: 123, 133, 162, 328,

0302-4369/84 $2.50

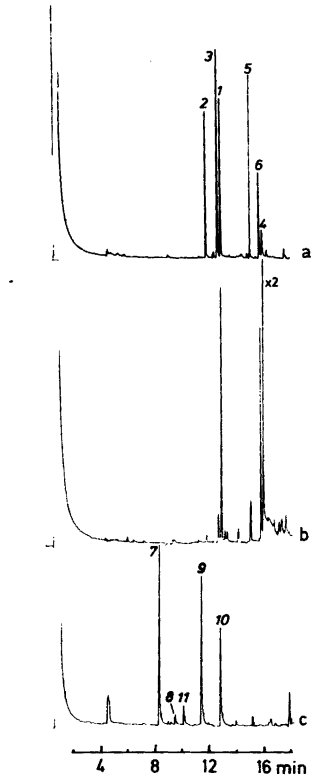


Fig. 1. Capillary gas chromatograms of (a) an equimolecular mixture of compounds *1–6*, (b) the fluorescent fraction from an extract of flowers of *C. coccineum* after prepurification on TLC and (c) as (b) with the whole plant of *T. erecta*.

2: 123, 133, 168, 316, *3*: 123, 133, 164, 330, *4*: 107, 133, 161, 372, *5*: 167, 212, 329, 360, *6*: 107, 135, 163, 374, the latter in each case being the molecular ion. This method, which in this case would allow detection of pyrethrin concentrations as low as 0.0001 %, did not furnish any evidence of the presence of pyrethrins in parts of plants or calli of the two *Tagetes* species. From extracts of roots of *T. erecta* and *T. patula* we isolated three thiophenes by means of preparative TLC. These were identified as *7*, *9* and *10* by 400 MHz ^{1}H NMR and MS. In addition, *8* and *11* have been identified from flower extracts. Compounds *7* and *10* are predominant in the root, while *9* predominates in the flowers. However, although all of these thiophenes are known, *11* does not seem to have been previously reported from *Tagetes* species.[6,7] In extracts of calli we have not been able to detect *8*.

Although, judging from TLC alone, it would seem that the *Tagetes* species contain pyrethrins, capillary GLC, ^{1}H NMR and MS provide the extra information necessary to show unequivocally that this is not true. Thus, it would appear that previous workers have mistaken thiophenes for pyrethrins. At present we have not been able to obtain samples of *Achillea ageratum*. However, our analyses of *A. millefolium* and *A. ptarmica* did not indicate even traces of pyrethrins.

Experimental. GLC analyses were performed with a Hewlett-Packard 5730A instrument equipped with FID, a 20 m × 0.33 mm BP-5 capillary column and HP 3385A automation system. Samples(2μl) were injected at 100 °C and the temperature was linearly raised by 8 °C/min to 244 °C. H_2 was used as carrier gas at a flow rate of 2 ml/min (100 °C). Flow rates for detector were 30 ml/min (H_2) 300 ml/min (air). Injector and detector temperatures were both set to 250 °C. GLC–MS analyses were carried out with Hewlett-Packard 5985A with a computer controlled quadrupole mass-analyzer and using EI at 70 eV. Samples (2μl) were injected at 100 °C on a similar column as the one above and the temperature program was: 100 °C–20 °C/min – 200 °C–4 °C/min–264 °C. 400 MHz ^{1}H NMR spectra were recorded on a Bruker WM-400 with $CDCl_3$ as solvent. Pyrethrum extracts (25 % in mineral oil) were purchased from Fluka and *Norsk Medisinal Depot.*

Preparation of standard solution of pyrethrins. Isolation of the pyrethrins was carried out according to Refs. 9 and 10. Yields from 100 mg of mixture freed from mineral oil were: *1* (26 mg), *2* (5 mg), *3* (2 mg), *4* (27 mg), *5* (3 mg) and *6* (4 mg). A standard solution containing 0.05 mg/ml of each of the compounds *1–6* was prepared. For quantitative GLC, methyl linoleate was used for *1–3* and methyl arachidate for *4–6* as internal standards.

Isolation and purification of thiophenes 7,9 and 10. Fresh roots of *Tagetes erecta* (160 g) were treated in a homogenizer with light petroleum (3×200 ml) followed by diethyl ether–light petroleum (1:10) (2×150 ml). The combined extracts were concentrated *in vacuo* and the remaining oil purified by TLC,[11] yields: *7* (15.4 mg), *9* (4.6 mg) and *10* (11.6 mg). The three thiophenes were identified by ^{1}H NMR (see Ref. 5). A mixture of *7*, *9* and *10* was used as standard for GLC.

Extraction, work up and GLC analysis of plants and calli. Dried plants were ground and extracted with hexane (3×100 ml/10 g dry weight). Most of the solvent was removed *in vacuo* and the remaining solution was extracted

with nitromethane (3×20 ml). Nitromethane was removed *in vacuo* and the remaining oil was applied to preparative TLC plates (0.5 mm, Merck kieselgel HF 60). Upon elution with hexane–ethyl acetate (75:25), two fluorescent bands were collected for both *Tagetes* species (R_f 0.29–0.38, 0.49–0.53) and for the prepurified Pyrethrum extract and *C. coccineum* (0.27–0.36, 0.40–0.52). After rechromatography and GLC–MS six pyrethrins and five thiophenes were identified. The thiophenes were identified as *7, 8, 9, 11* (upper band) and *10* (lower band). The pyrethrin I-group (*1–3*) was found in the upper band while the II-group (*4–6*) was found in the lower band.

Hypocotyl calli of *Tagetes erecta* grown under the same conditions as in Ref. 3 were extracted with acetone without drying. Subsequent treatment as above and GLC–MS showed small, but significant, amounts of *7, 9* and *10*. Typical yields in % of dry weight of callus: $0.1 \cdot 10^{-4}$ (*7*), $1 \cdot 10^{-4}$ (*9*) and $8 \cdot 10^{-4}$ (*10*).

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Received September 3, 1984.