# ANALYSIS OF THIOPHENES IN THE TAGETEAE (ASTERACEAE) BY HPLC

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ABSTRACT.—The distribution and ontogenetic changes in four biosynthetically related thiophenes from hydroponically grown *Tagetes patula* L. were investigated using reverse-phase hplc. Four thiophenes, 5-(4-hydroxy-1-butenyl)-2,2'-bithienyl (**1**), 5-(4-acetoxy-1-butenyl)-2,2'-bithienyl (**2**), 5-(3-buten-1-ynyl-2,2'-bithienyl (**3**) and 2,2':5'2"-terthienyl (**4**) were identified from all parts of the plants, their relative concentrations varying between root, shoot and flower. Compound **3** was predominant in roots while **2** was the dominant derivative in shoots. Flowers contained compounds 2–4 along with as yet unidentified compounds with uv absorption spectra characteristic of thiophenes. Compounds **2** and **3** in roots and **2** in shoots increased over the life of the plant, but their concentration was found to stabilize following flowering.

The presence of these four thiophenes in other members of the tribe Tageteae was also examined. Some or all of the compounds were found in three species of *Dyssodia*, one species of *Porophyllum* and nine species of *Tagetes*. Only *Pectis* species lacked all four of the thiophenes.

Thiophenes comprise a distinct class of natural plant products characteristic of the Asteraceae (5). These secondary compounds possess one, two or three aromatic, five-membered, sulfur-containing rings linked together by alpha carbons (fig. 1). Two of them, 5-(3-buten-1-ynyl)-2,2'-bithienyl (**3**) and $\alpha$-terthienyl

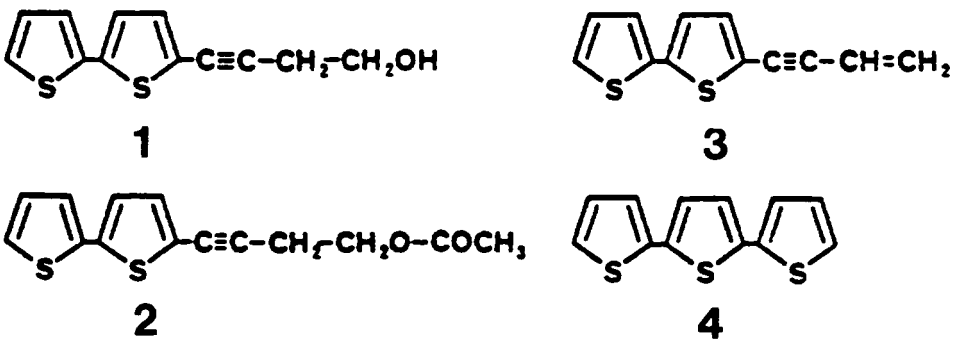


FIGURE 1. Chemical structures of the four naturally occurring thiophenes examined.

(**4**), were first isolated from petals of the "African" variety of the common marigold (*Tagetes erecta* L.; Tribe Tageteae) (1,2). Other biosynthetically related compounds including 5-(4-hydroxy-1-butenyl)-2,2'-bithienyl (**1**) (3) and 5-(4-acetoxy-1-buten)-2,2'-bithienyl (**2**) (4) were also isolated from members of the Tageteae. To date thiophenes have been found in a total of thirteen species of *Tagetes* (3–6), two species of *Dyssodia* (7) and one species of *Porophyllum* (3). They also have been reported to occur in nine other tribes of the Asteraceae (8) and thus represent natural products with a wide distribution.

The photobiocidal effects associated with one of the thiophenes, $\alpha$-terthienyl, are well documented (13–17). The potent nematocidal properties of this compound, originally described by Uhlenbroeck and Bijloo (9,10), were subsequently demonstrated to be enhanced in near ultraviolet light (320–400nm) (11,12). Chan *et al.* (13) reported that both **3** and **4** were "phototoxic" to bacteria and yeast in UV–A, but were not lethal in the dark. In addition to nematodes, bacteria and yeast, the effects of these compounds have been demonstrated with algae (14), insects (15, 16) and various other test organisms (17).

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Previous studies have shown roots to be a major source of compounds **3** and **4** (18). Leaves (5), flower petals (1,2), and the pappus of achenes (13) also have been found to contain low concentrations of these derivatives. Studies involving the distribution, levels of accumulation and ontogenetic changes associated with these compounds have not been feasible so far because of the lack of a sensitive method capable of rapid separation and detection. This study was undertaken to develop a rapid analytical system based on reverse-phase high performance liquid chromatography (hplc) which could be used for the separation and quantitation of these unstable thiophenes from relatively crude plant extracts. With this technique, the composition of thiophenes in *T. patula* L. as well as of eighteen other representatives of the tribe Tageteae were studied.

## MATERIALS AND METHODS

CHEMICALS.—5-(4-hydroxy-1-butenyl)-2,2'-bithienyl (**1**) and 5-(4-acetoxy-1-butenyl)-2,2'-bithienyl (**2**) were kindly supplied by Dr. R. Süetfeld, University of Münster. Compound **3**, 5-(3-buten-1-ynyl)-2,2'-bithienyl, was isolated from the roots of either *Tagetes patula* L., *T. erecta* L. or *Dyssodia papposa*. Alpha-terthienyl (**4**) was isolated from the same plant sources or supplied by Dr. F. Garcia, Chemistry Department, National University of Mexico. Reference compounds were checked for purity by hplc and glc-ms and spectroscopically identified by ir, uv, nmr and ms. Mass spectral analyses were kindly provided by the Chemistry Department at Simon Fraser University, Burnaby, B.C.

PLANT MATERIAL.—Achenes of *T. patula* L. (J. L. Hudson, Redwood City, California) were used for all experiments. The achenes were germinated in glass petri dishes on Whatman #1 filter paper moistened with distilled water. Uniform seedlings approximately 5 cm long were selected at 4 to 5 days and transferred to well-aerated, one-tenth strength Johnson's nutrient solution (20). The hydroponic seedlings were kept in growth chambers (Coviron, Controlled Environments Ltd., Winnipeg, Manitoba, Canada) at 25°, 100% R.H. with a photoperiod of 16h light/8h dark. The nutrient solution was changed on a weekly basis.

Dried plants used for the examination of thiophenes in the tribe Tageteae were obtained from various sources. *Pectis filipes* var. subnuda (K13230), *P. imberbis* (13559, 13566), *P. longipes* (diploid; K11375, 13500, 13508 and tetraploid; K11368, 13529, 13535), *P. papposa* var. papposa (K13149), and *P. prostrata* (K13279) were supplied by Dr. David J. Keil, California Polytechnic University, San Luis Obispo, California. *Tagetes multiflora* (389a,b) and *T. ellipticum* (112) were collected by Timothy Johns, University of Michigan, Ann Arbor, Michigan. *Dyssodia anthemidifolia, D. decipiens* (58), *D. papposa, Porophyllum gracile, Tagetes coronopifolia* (105), *T. filifolia* (79), *T. lunulata, T. lucida* (111), and *T. tenuifolia* (106) were collected by one of us (GHNT). *Tagetes lemmonii* (University of California Botanical Garden 74,251) was supplied by Dr. John Strother, Herbarium, University of California, Berkeley, California. *Tagetes lunulata, T. patula, T. erecta* were grown in the field from seed. Voucher specimens were deposited in the Botany Department Herbarium, The University of British Columbia, Vancouver, B.C., Canada.

EXTRACTION OF THIOPHENES.—Separated roots or shoots of *T. patula* were extracted in methanol by grinding the fresh tissue in a Waring blender (3x) following the procedure of Chan *et al.* (18). The brei was filtered and the combined extracts diluted with an equal volume of distilled water and extracted (3x) with equal volumes of petroleum ether (30–60°). The petroleum ether fractions were dried overnight with anhydrous $Na_2SO_4$. The extract was evaporated to dryness by rotary evaporation and the residue immediately resuspended in hplc grade methanol and chromatographed.

Dried plant material and herbarium specimens were extracted by soaking 1–5g of powdered material into 20ml of 95% ethanol for 3 to 4 weeks. The dry material was removed by filtration and concentrated by rotary evaporation to approximately 1–2ml prior to hplc examination.

Leaf glands were ruptured with capillary pipettes. The oil was collected by capillary action and transferred into hplc grade MeOH by forcing air through pipettes. Approximately 800–1000 glands were sampled from several plants. All extraction procedures were conducted under dimmed room lights to avoid photodegradation of the extracted components. Extracts were stored at −20° until analysis.

QUALITATIVE AND QUANTITATIVE ANALYSES.—Qualitative and quantitative analyses of thiophenes were carried out on a Varian Series 5000 LC. A Variscan 634 S spectrophotometer set to 350nm was used to detect eluted compounds which were separated on Varian MicroPak MCH-10 (4 x 30mm) octadecylsilane reverse-phase columns. An isocratic solvent system consisting of acetonitrile and water (72:28) containing 10mM each of K $P_i$ buffer (pH 3.2) and tetramethylammonium chloride (Aldrich Chemical Co.) was used to elute thiophenes following the method of Phillips and Towers (21). All hplc was conducted at room temperature with flow rates of 1 ml $min^{-1}$.

For quantitation of thiophene derivatives, stock solutions of each standard were prepared in 95% ethanol. To determine calibration curves, the 20$\mu$l injection loop was filled with various diluted standards, and the resulting peak areas were measured from the chart recorder. At least five injections of each dilution were made. To determine peak areas, the peak height was multiplied by the width at half height. All values were corrected for recorder sensitivity. The data was analyzed by linear regression and the line of best fit used for concentration

determinations from crude extracts. All calibration curves had coefficient of determination values ($r^2$) of 0.99 or better.

Two studies involved quantitative hplc. The first involved the determination of thiophene levels in mature, flowering *T. patula* plants. Concentrations of the thiophenes were determined for roots, shoots (stems and leaves), immature leaves, mature leaves and flowers.

In a second study, the ontogenetic changes in thiophene concentrations from early seedling to flowering were examined. Approximately 45 *T. patula* seedlings were grown under hydroponic conditions. Five plants, selected randomly, were harvested at two to three week intervals. The plants were frozen at −80°, lyophilized and extracted by Soxhlet extraction with petroleum ether. The experiment was repeated three times, and standard deviations were found to vary by less than 5%.

## RESULTS AND DISCUSSION

Reverse-phase hplc was used for both qualitative and quantitative analyses of the thiophenes in extracts of *Tagetes*. Table 1 lists the retention times ($t_R$),

TABLE 1. Retention times ($t_R$), adjusted retention times ($t'_R$), separation factors ($\alpha$) and resolution values (R) of thiophene standards separated by hplc[a] ($t_o$=1.9 min).

| Thiophene | $t_R$ | $t'_R$ | $\alpha$ | R |
|---|---|---|---|---|
| 1 | 5.6 | 3.5 | | |
| 2 | 6.9 | 5.0 | 1.43 | 3.53 |
| 3 | 9.1 | 7.2 | 1.44 | 4.00 |
| 4 | 10.8 | 8.9 | 1.24 | 3.09 |

[a]hplc parameters defined in reference (25).

adjusted retention times ($t'_R$), separation factors ($\alpha$) and resolution values (R) of the thiophene reference compounds. Elution from the column was in the order of decreasing polarity. Compound **1**, the most polar derivative, was retained least ($t_R$=5.6 min) and was eluted before the more non-polar thiophenes **2** ($t_R$=6.9 min), **3** ($t_R$=9.1 min) and **4** ($t_R$=10.8 min).

QUALITATIVE ANALYSIS.—Chromatographic examination of the extracts from various parts of 8-week old *T. patula* plants revealed differences in thiophene patterns (fig. 2a-d). The compounds absorbing at 350 nm which were extracted from flowers are shown graphically in fig. 2a. Alpha-terthienyl (**4**) was the major known thiophene derivative although **2** was well represented. Two unidentified peaks with $t_R$ values of 8.3 and 12.0 min were also present in substantial amounts. These unidentified peaks showed uv absorption spectra which were similar to those of other thiophenyl compounds and may, therefore, be thiophene derivatives. Only two peaks were detectable in the oil of the glandular trichomes (fig. 2b). They were found to correspond to compound **3** plus the unidentified component also found in aerial plant parts.

Shoot (stems and leaves) and root extracts contained thiophenes **1**–**4** (fig. 2c and d). Their relative proportions were quite different from those found in flowers. An unidentified peak with a $t_R$ of 8.3 min was a major shoot component, but appeared in much lower amount in roots.

QUANTITATIVE ANALYSIS.—Roots showed the highest concentrations of **2** and **3** (table 2). Alpha-terthienyl (**4**) was prevalent in flowers, whereas compound **1** was found to be only a minor component throughout the plant. Growing leaves contained considerably higher levels of thiophenes than mature tissue. Differences between older and younger leaf tissue may be a result of the loss of compounds by: 1) biochemical or photochemical degradation in older tissue; 2) transport from older to younger tissue; 3) volatilization from glands; or 4) dilution in older tissue. Conversely, younger tissue may be the site of thiophene synthesis.

The fate of thiophenes over the life of the plant was also investigated quantitatively. Fig. 3a shows the level of **2**, **3** and **4** from root extracts of hydroponically

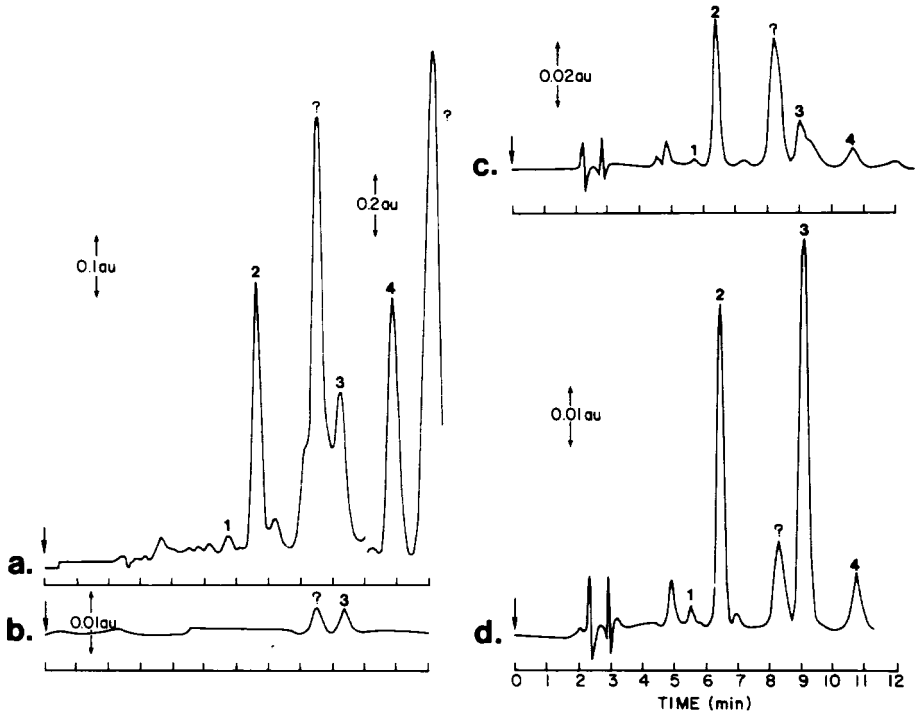


FIGURE 2. HPLC chromatograms of compounds absorbing 350nm irradiation which were extracted from flowers (a), leaf oil glands (b), shoots (c) and roots (d) of eight-week old *Tagetes patula* L. plants grown hydroponically. HPLC conditions are given in *Materials and Methods*.

grown plants. Compounds **2** and **3** were the main thiophenes during early seedling growth increasing in concentration for the first 80 days after germination and reaching a plateau after that time. The concentration of **4** changed very little over the experimental period.

TABLE 2. Thiophene levels in crude extracts of 75-day-old hydroponically grown plants. Standard deviation of three injections are listed in parentheses. All concentrations are in micromoles per gram fresh weight (uM $g^{-1}$ fr wt).

| Compound | Root | Shoot | Immature Leaf | Mature Leaf | Flower |
|---|---|---|---|---|---|
| **1** | 0.2(0.0) | 0.2(0.0) | 0.3(0.1) | — | — |
| **2** | 12.3(0.3) | 6.4(0.2) | 1.5(0.3) | 0.6(0.1) | 0.5(0.1) |
| **3** | 26.6(1.1) | 0.6(0.1) | 0.4(0.0) | — | 0.4(0.1) |
| **4** | 0.5(0.2) | 0.5(0.1) | 0.5(0.1) | — | 1.3(0.2) |

Compound **2** was the dominant thiophene in shoots (fig. 3b). Derivatives **3** and **4** were equally concentrated and did not increase appreciably over time. Compound **2**, on the other hand, increased until around 75–80 days and then levelled off. The levelling of **2** in the shoot and **2** and **3** in the roots followed flowering quite closely.

The significance of the distribution and accumulation of the various thiophenes within different plant parts is not as yet clear. It is tempting to speculate, however, that the light-induced nematicidal (11,12), insecticidal, (15) and allelopathic (26) activities which have been demonstrated for **4** may also be associated with multiple defensive roles of all of the various derivatives throughout

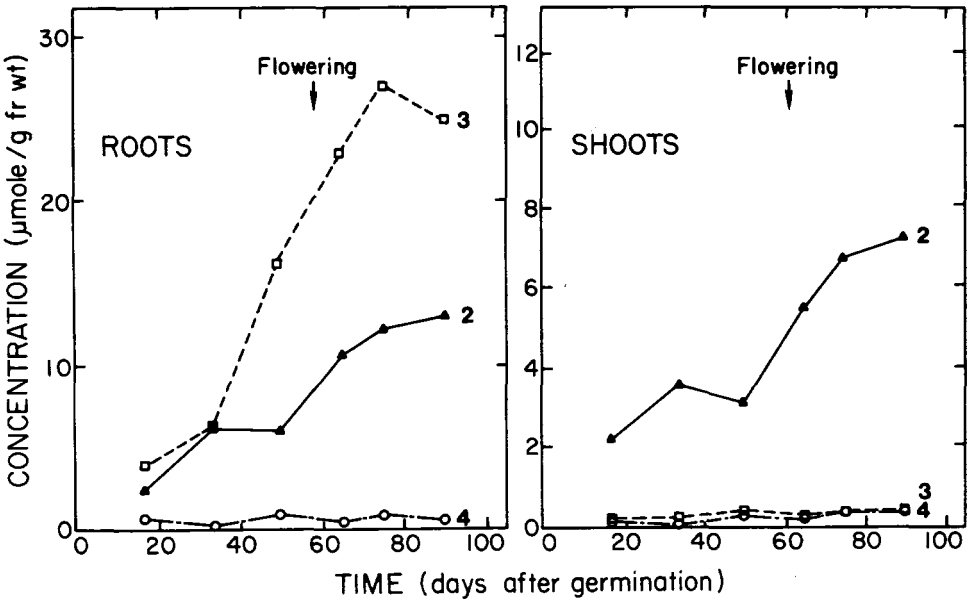


FIGURE 3. Ontogenetic changes in concentrations of 2 (△), 3 (□) and 4(○) from hydroponically grown *Tagetes patula* L. plants. Each point represents the average of at least three injections of an extract. All extractions were made from the pooled roots or shoots from five plants.

the life of the plant. Accumulation of these allelochemicals during vegetative growth further suggests that these compounds may need to increase to levels sufficient to protect the plant from herbivore attack prior to and during flowering.

DISTRIBUTION OF THIOPHENES IN THE TAGETEAE.—Eighteen species representing four of the largest genera of the tribe Tageteae were examined for the presence of thiophenes **1–4**. Table 3 shows that the bithienyl and terthienyl derivatives

TABLE 3. Distribution of thiophenes within the tribe Tageteae examined by hplc.

| Plant Species | Thiophenes | | | |
|---|---|---|---|---|
| | **1** | **2** | **3** | **4** |
| *Dyssodia anthemidifolia* Benth. | | | + | + |
| *D. decipiens* Bartl. | + | + | + | + |
| *D. papposa* (Vent.) Hitchc. | + | + | + | + |
| *Pectis filipes* Harv. & Gray | | | | |
| *P. imberbis* A. Gray | | | | |
| *P. longipes* A. Gray | | | | |
| diploid | | | | |
| tetraploid | | | | |
| *P. papposa* Harv. & Gray | | | | |
| *P. prostrata* Cav. | | | | |
| *Porphyllum gracile* Benth. | + | + | + | + |
| *Tagetes coronopifolia* Willd. | + | + | + | + |
| *T. erecta* L. | + | + | + | + |
| *T. filifolia* A. Gray | | + | | |
| *T. lemonii* A. Gray | + | + | + | + |
| *T. lunulata* Ort. | | + | + | + |
| *T. minuta* L. | + | + | + | + |
| *T. multiflora* HBK. | | | + | + |
| *T. patula* L. | + | + | + | + |
| *T. tenuifolia* Cav. | + | | + | + |

were common in the genera *Dyssodia*, *Porophyllum*, and *Tagetes*. Thiophenes, however, were totally lacking in species of *Pectis*.

Based on morphological characters, *Pectis* has no close relatives among other genera of the tribe (22–23), and the chemical uniqueness of the genus is further reflected by the fact that 20 out of approximately 80 species thus far examined display the Kranz syndrome or $C_4$ metabolism (24). The absence of thiophenes shown in our study may be further evidence that *Pectis*, the largest genus in the Tageteae, is distantly related to other members of the tribe.

These preliminary results show reverse-phase hplc to be a powerful analytical tool for the analysis of thiophenes in relatively crude plant extracts and should prove a valuable technique for further studies of the *Tageteae* and the *Asteraceae*. The presence of these photosensitizers in shoot systems pose a number of interesting questions concerning their biological significance. For instance, is their "phototoxicity" expressed *in situ* in defense of the plant? If so, how do plants avoid autotoxicity?

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