

Arrhenius parameters are given and may allow prediction of shelflife as well as stability in biological media at various temperatures and conditions. Salt effects are also discussed and point toward reaction between two oppositely charged ions. A diagram of the entire process is shown in Scheme II.

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## Isolation and Identification of Three New Flavones from *Achillea millefolium* L.

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**Abstract** □ Column chromatography on silica gel of a petroleum ether extract of the flowering heads of *Achillea millefolium* L. allowed three flavones to be separated and identified. Spectral studies (PMR, mass spectrometry, and UV) and a comparison with data for compounds reported in the literature established the flavones as 5-hydroxy-3,6,7,4'-tetramethoxyflavone, artemetin, and casticin. These compounds have not been reported previously as constituents of *A. millefolium*.

**Keyphrases** □ Flavones—chromatographic separation and spectral identification from *Achillea millefolium* □ *Achillea millefolium*—chromatographic isolation and spectral identification of flavone components □ Medicinal plants—*Achillea millefolium*, isolation and identification of flavone components

*Achillea millefolium* L. (Compositae), commonly known as yarrow, grows abundantly throughout America and Europe (1). The plant has been a popular medicinal remedy, having been known to Dioscorides (2), and has been recommended for use as a tonic, a stomachic, a hemostatic, an antispasmodic, an antihemorrhoidal, an emmenagogue, an antiseptic, and an anthelmintic (3).

Chemical investigations of the plant have been numerous, extending back for more than 200 years (4, 5). One class of compounds that has received considerable attention is the flavonoids; they have been proposed as the cause of the spasmolytic activity of *A. millefolium*. Horhammer (6) examined a number of flavones and extracts of different plants for antispasmodic activity and showed *A. millefolium* to be the most active plant. He found that the plant contained apigenin and luteolin-7-glycosides. These flavones, as well as caffeic acid, were found also by Michaluk (7), and the presence of caffeic acid was more

recently verified (8). In 1964, luteolin-7-glucoside was isolated from *A. millefolium* and synthesized (9). More recently, the presence of isorhamnetin was reported (10).

The present work yielded three polymethoxy flavones not previously reported as constituents of this plant.

#### EXPERIMENTAL

**Plant Material**—The flowering heads of *A. millefolium* were harvested in June 1970 at the University of Illinois Drug and Horticultural Experimental Station, Downers Grove, Ill. The seeds of wild growing *A. millefolium* were collected in the Bemis Woods, McHenry County, Ill., and were planted in a greenhouse at the Station. Shoots were transplanted from one generation to the next to assure that material of the same species was maintained. Only the inflorescence of the plant was used and was harvested at the time of opening of the flowers. The plant was identified as *A. millefolium* by chromosome count<sup>1</sup>.

**Extraction**—One kilogram, 500 g at a time, of air-dried, coarsely ground plant material was extracted in a soxhlet extractor. Each 500-g portion was extracted for 24 hr with 3400 ml of petroleum ether, the extract was removed, 2500 ml of fresh solvent was added, and the extraction was continued for an additional 48 hr. All solvents were combined, and an insoluble precipitate, F1, was filtered off and set aside. The filtrate was condensed by evaporation to 1500 ml and again filtered, yielding another precipitate, F2.

The precipitate F1 was crystallized from ligroin, and the filtrate was combined with F2. The crystals were triturated with ether, and the filtrate again was added to F2. Finally, the crystals were recrystallized from chloroform-ether to yield 3.75 g of achillin, a sesquiterpene lactone previously isolated from this plant (11), and this filtrate was combined with F2.

<sup>1</sup> Performed by Dr. Edward D. Garbner, Department of Botany, University of Chicago.

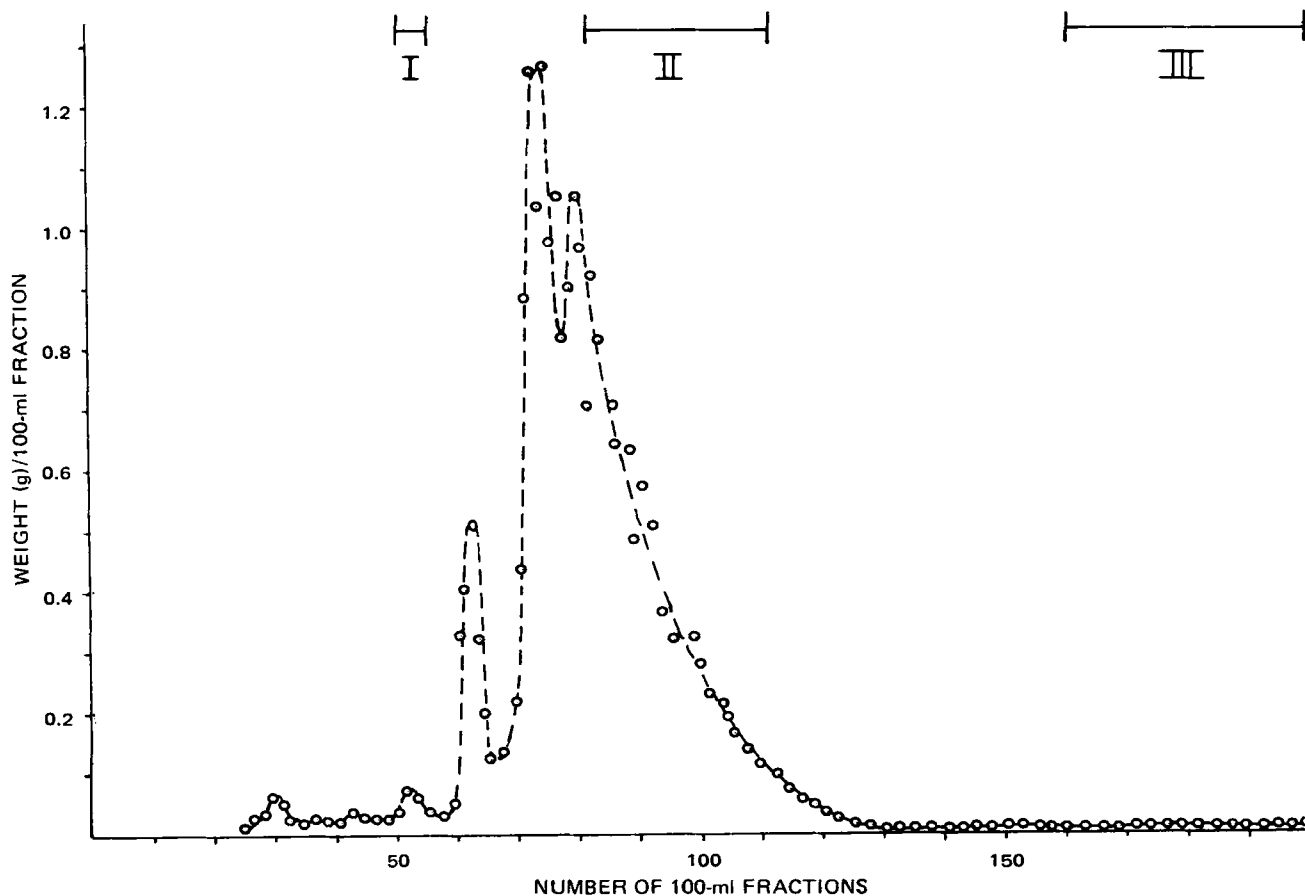


Figure 1—Residue weights versus column fraction number for chromatographic separation on silica gel.

Fraction F2, containing the flavonoids, was evaporated to dryness *in vacuo* (36.4 g). The fraction was chromatographed on a silica gel<sup>2</sup> column prepared by first activating the silica (1800 g) at 120° for 24 hr and then deactivating with 10% distilled water before preparing a 30% slurry in benzene–ethyl acetate (9:1). The column was allowed to settle for 24 hr before being charged, resulting in a column having the dimensions of 80 × 8 cm.

The fraction was dissolved in a minimum of eluent, benzene–ethyl acetate (9:1), and chromatographed. Two hundred column fractions of 100 ml each were collected, each was evaporated to dryness, and the residue weight was determined. A plot of the residue weight *versus* fraction number is shown in Fig. 1.

**Isolation of 5-Hydroxy-3,6,7,4'-tetramethoxyflavone (I)**—Column fractions 50–56 (Fig. 1) were combined and evaporated to dryness *in vacuo* (0.31 g). The residue was crystallized from ether, yielding the flavone I as pale-yellow crystals (0.142 g), mp 168–170° [lit. (12) mp 169–171°]; PMR<sup>3</sup> (deuteriochloroform with tetramethylsilane as the internal reference), ppm ( $\delta$ ): 3.87 (s, 3), 3.90 (s, 3), 3.92 (s, 3), 3.95 (s, 3), 6.49 (s, 1), 7.01–8.07 (m [A<sub>2</sub>B<sub>2</sub>], 4), and 12.60 (s, 1). UV<sup>4</sup> spectra in methanol and with various reagents are given in Table I. The mass spectrum<sup>5</sup> of I is given in Fig. 2.

**Isolation of Artemetin (II)**—As indicated in Fig. 1, fractions 78–109 were combined and evaporated to dryness *in vacuo*, yielding 11.84 g of residue. This residue consisted primarily of achillin, as shown by TLC and spectral data, with one minor component (<5%). This mixture was rechromatographed, using 300 g of basic alumina (deactivated with 5% water) on a 3 × 31-cm column, and eluted with 875 ml of chloroform–acetone (1:1) to remove the achillin. The column was then eluted with 625 ml of 1-butanol–acetic acid–water (4:1:1) to remove the flavone.

After evaporation to dryness *in vacuo*, a powdery residue of the compound and alumina remained. To remove the alumina, 200 ml

of benzene was added and the mixture was extracted twice with 200-ml portions of 0.1 N HCl. The aqueous layer was extracted twice with 200-ml portions of benzene, and the benzene layer was finally separated and evaporated to dryness *in vacuo*, leaving an orange residue. Recrystallization was from benzene–petroleum ether to yield a yellow-orange solid (0.070 g), mp 159–160° [lit. (13) mp 161.5°].

Table I—UV Spectral Data in Methanol with Various Reagents

Reagent	$\lambda_{\max}$ , nm (log $\epsilon$ )	
	Compound I	Compound III
None	213 (4.58)	212 (4.64)
	274 (4.41)	258 (4.36)
	338 (4.46)	270 (4.32)
Sodium methoxide	—	351 (4.38)
	223 (4.57)	214 (4.75)
	294 (4.52)	273 (4.45)
Aluminum chloride	323 (sh)	385 (4.15)
	213 (4.46)	214 (4.67)
	238 (4.18)	270 (4.39)
	282 (4.24)	383 (4.39)
Aluminum chloride–hydrochloric acid	305 (4.18)	—
	370 (4.43)	—
	212 (4.43)	213 (4.61)
	236 (4.16)	267 (4.37)
Sodium acetate	288 (4.26)	282 (4.35)
	358 (4.41)	373 (4.34)
	225 (4.49)	225 (4.64)
	273 (4.40)	258 (4.36)
Sodium acetate–boric acid	338 (4.49)	270 (4.32)
	—	351 (4.38)
	226 (4.49)	227 (4.59)
	274 (4.42)	258 (4.36)
	338 (4.49)	270 (4.32)
	—	351 (4.38)

<sup>2</sup> PF-254, E. Merck, Darmstadt, Germany.

<sup>3</sup> Varian Associates models A60 and T60A spectrometers.

<sup>4</sup> Perkin-Elmer model 202 recording spectrophotometer.

<sup>5</sup> Hitachi-Perkin-Elmer RMU-6D operating at 70 eV.

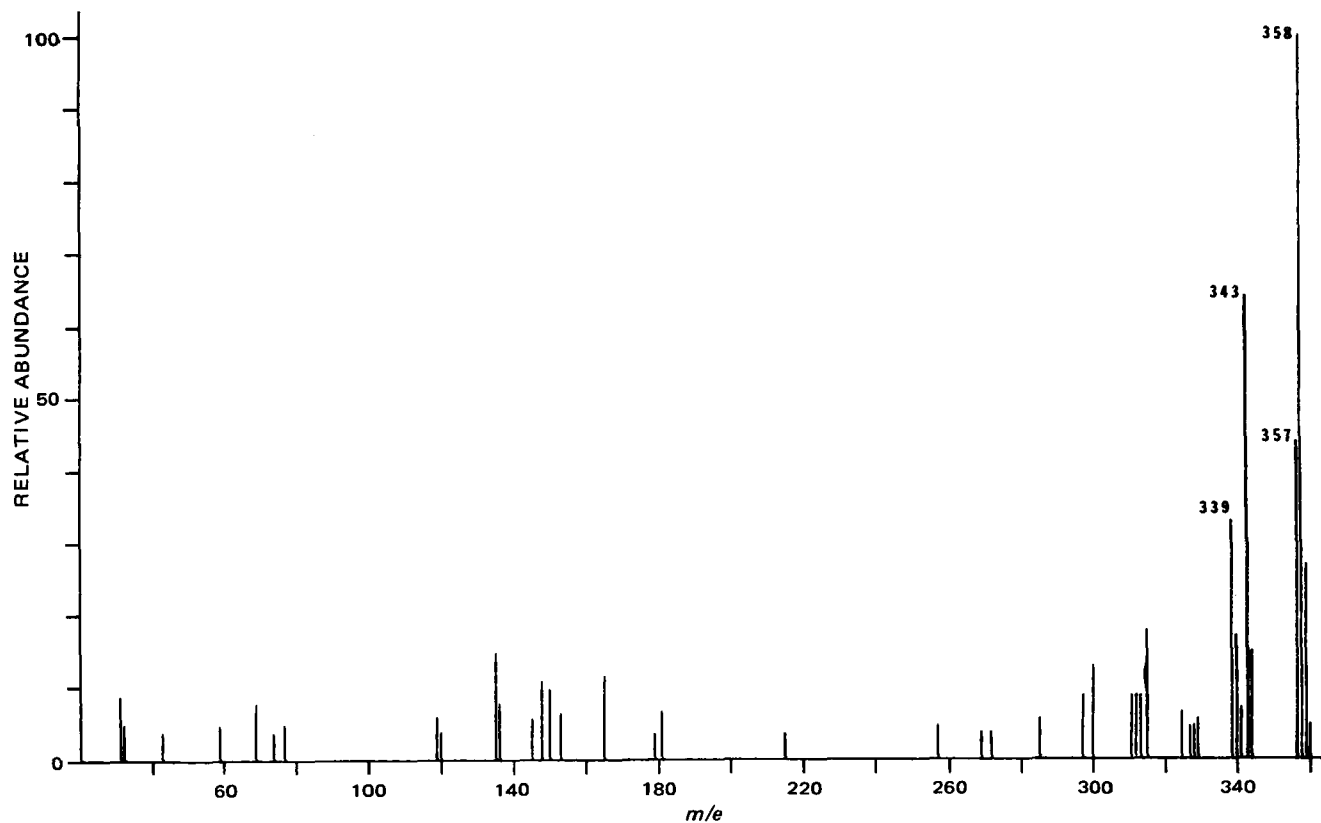


Figure 2—Mass spectrum of 5-hydroxy-3,6,7,4'-tetramethoxyflavone at 70 ev.

The mass spectrum of II is given in Fig. 3. Other spectral data already appear in the literature (13, 14).

**Isolation of Casticin (III)**—Column fractions 157–200 (Fig. 1) were combined and evaporated to dryness *in vacuo* to yield a yellow solid (0.25 g). This solid was dissolved in 50 ml of chloroform, extracted twice with 20-ml portions of 5% NaOH, and separated.

The pH of the aqueous phase was adjusted to 8 by bubbling carbon dioxide gas through the solution, causing a yellow-green precipitate to form.

The solid was separated by centrifugation and dried (0.090 g), mp 183–185° [lit. (15, 16) mp 186–187°]; PMR<sup>3</sup> (deuteriochloroform with tetramethylsilane as the internal reference), ppm ( $\delta$ ):

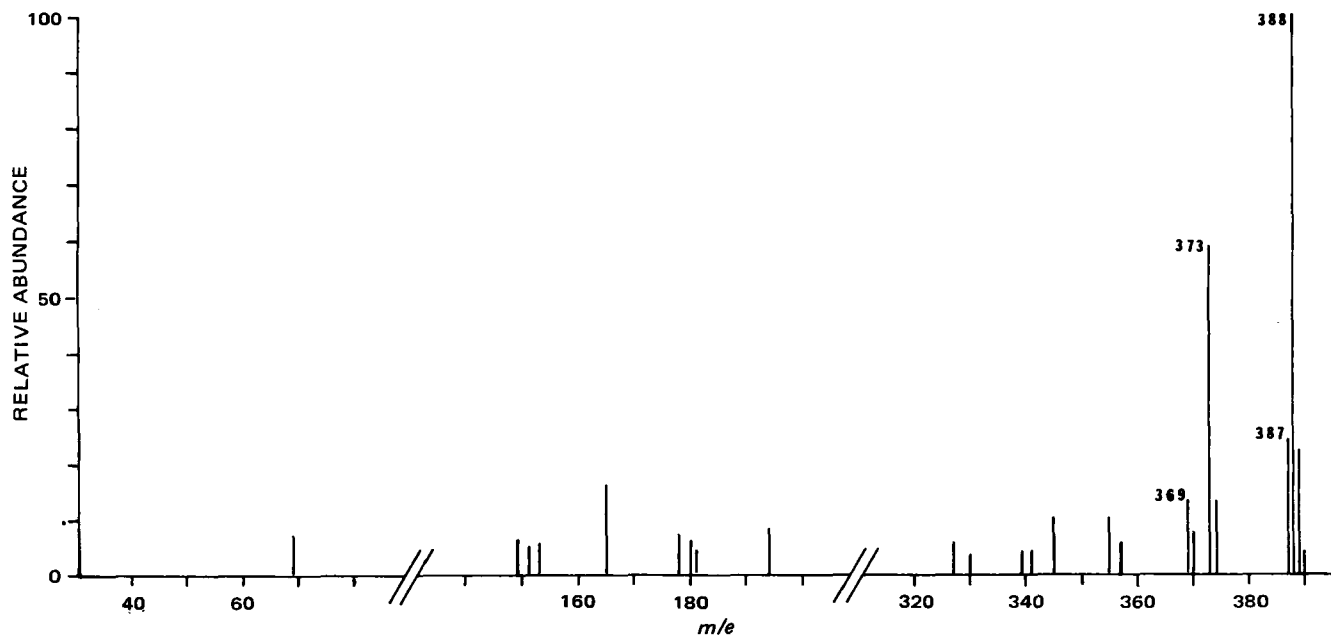


Figure 3—Mass spectrum of artemetin at 70 ev.

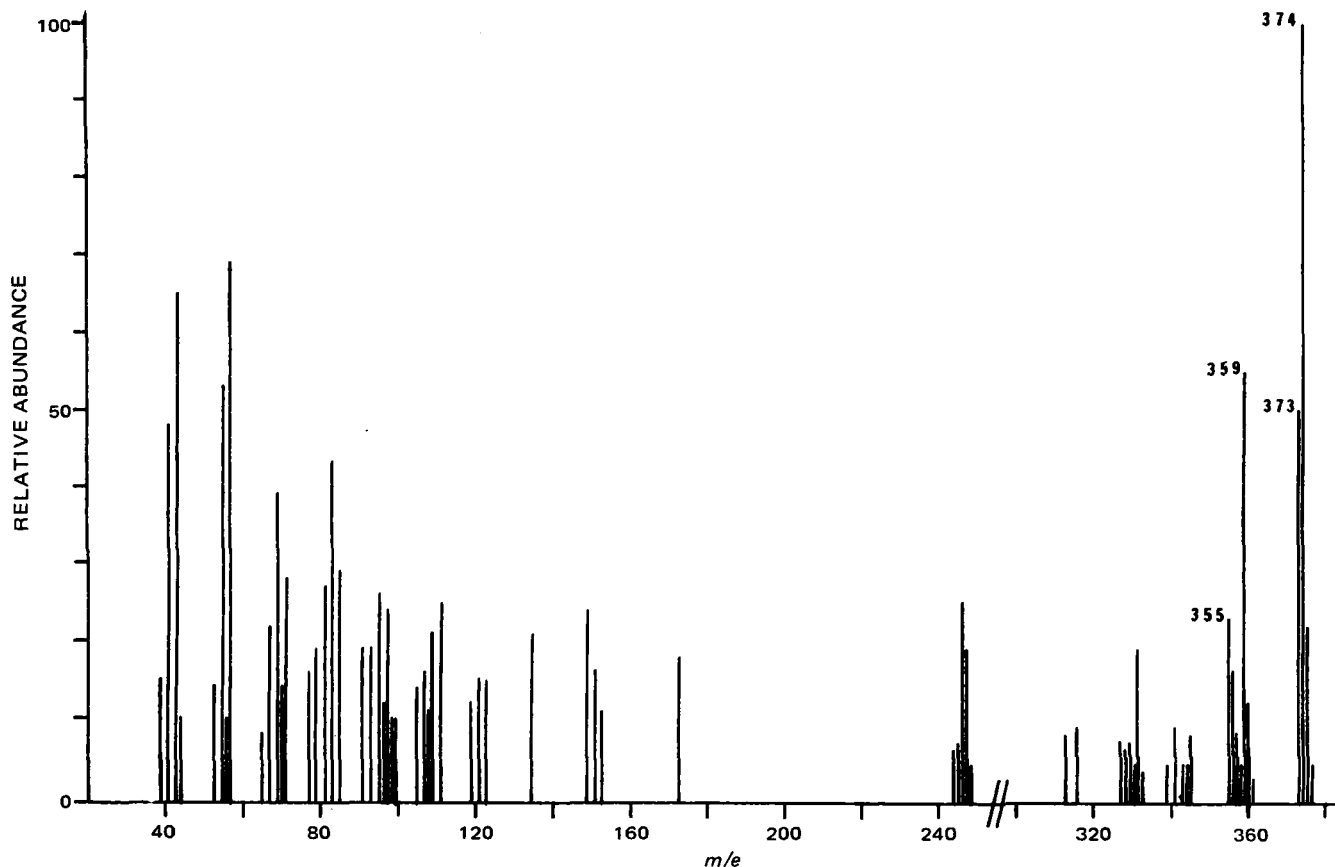


Figure 4—Mass spectrum of casticin at 70 eV.

3.87 (s, 3), 3.92 (s, 3), 3.95 (s, 3), 3.98 (s, 3), 6.49 (s, 1), 6.95 (d, 1,  $J = 9.6$  Hz), 7.65 (d, 1), 7.69 (dd, 1,  $J = 9.6$  Hz,  $J = 2.0$  Hz), 12.58 (s, 1), and 5.76 (s, 1). UV<sup>4</sup> spectra in methanol and with various reagents are given in Table I. The mass spectrum<sup>5</sup> of III is shown in Fig. 4.

## RESULTS AND DISCUSSION

Three flavonoids were isolated from the petroleum ether extract of the inflorescence of *A. millefolium* and proved to be 5-hydroxy-3,6,7,4'-tetramethoxyflavone (I), artemetin (II), and casticin (III).

Compound I was purified by column chromatography and identified by its physical and spectral properties. The mass spectrum showed both a parent peak at  $m/e$  339 (base peak) and a large  $P - 1$  fragment ion, characteristic of flavones, at  $m/e$  357.

A prominent ion at  $m/e$  343, representing a methyl loss from the parent ion, and a peak at  $m/e$  339, attributed to the loss of water from the  $P - 1$  fragment, also were observed. An expected ion in flavone spectra arising from a retro Diels-Alder fragmentation of the heterocyclic ring was not observed, and its absence was explained by the substitution at the 3-position on the flavone nucleus (17). This conclusion was corroborated by the similarity in the mass spectra of the other isolated flavones, which also bear a sub-

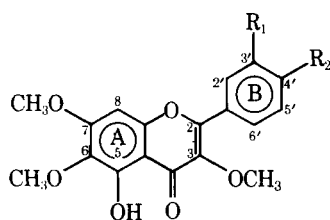
stituent at the 3-position. Stabilization of the heterocyclic ring by the loss of a methyl radical to produce an oxonium ion (IV) probably occurred in each instance to produce a stable even-electron ion.

The IR spectrum<sup>6</sup> revealed two strong absorptions at 1650 and 1600  $\text{cm}^{-1}$ , indicating an  $\alpha,\beta$ -unsaturated ketone with extended conjugation (18).

The 60-MHz PMR spectrum contained four singlets (3H each), indicating four methoxy groups at  $\delta$  3.87, 3.90, 3.92, and 3.95. The singlet at  $\delta$  6.49 (1H) represented an isolated aromatic proton (8-position), and the  $A_2B_2$  pattern at  $\delta$  7.01 (2H) and 8.07 (2H) indicated a *para*-substituted phenyl group. The remaining downfield singlet (1H) at  $\delta$  12.60 was highly characteristic of a C-5 phenolic hydroxy proton hydrogen bonded to the pyrone carbonyl (19).

From these spectral data, a monohydroxy tetramethoxyflavone with a molecular formula of  $C_{19}H_{18}O_7$  was concluded. While the PMR spectrum gave some information on the substitution pattern on the flavone nucleus, the precise arrangement of substituents was verified by the method of Mabry *et al.* (14) by observing the effect of a series of reagents on the UV spectrum in methanol.

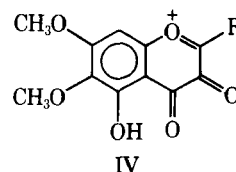
The UV maximum observed in methanol was indicative of a flavone with oxygen substitution in the A ring. The bathochromic shift noted upon addition of sodium methoxide indicated that a hydroxy group was present but not on the 3- or 4'-position. The spectrum in the presence of aluminum chloride fixed the hydroxy substituent at the 5-position, and the addition of hydrochloric acid



I:  $R_1 = H$ ,  $R_2 = OCH_3$

II:  $R_1 = R_2 = OCH_3$

III:  $R_1 = OH$ ,  $R_2 = OCH_3$



<sup>6</sup> Perkin-Elmer models 337 and 700 spectrophotometers. Solution spectra were obtained in deuteriochloroform, using 0.15-mm KBr cells.

to the previous solution caused a shift of 20 nm, considered diagnostic of a 5-hydroxyflavone having a methoxy group on the adjacent 6-position.

These deductions, coupled with the PMR pattern reported, fixed the position of all but two methoxy groups with permissible ring sites limited to the 3-, 7-, and 8-positions. Chemical shift data fixed the substituents at the 3- and 7-positions, since the singlet at  $\delta$  6.49 was characteristic of a C-8 proton with a nonhydroxy substituent at the 7-position (14). Furthermore, the chemical shifts observed for the B ring protons were consistent with an oxygenated 3-position (14, 20).

The structure assignment of 5-hydroxy-3,6,7,4'-tetramethoxyflavone was verified by a comparison with literature data, since the compound had been isolated from *Dodonaea labulata* (20) and synthesized by Quijano *et al.* (12).

The flavone II was more readily identified, since its PMR and UV spectra appeared in the literature (13, 14). The mass spectrum (Fig. 3) exhibited a molecular ion at  $m/e$  388 and the same loss of 1 and 15 mass units observed for I. In addition, a peak at  $m/e$  369 corresponded to the water loss from the P - 1 fragment ion mentioned previously. A 220-MHz PMR spectrum<sup>7</sup> and the series of six UV spectra (using the reagents shown in Table I) were identical with those published for artemetin (5-hydroxy-3,6,7,3',4'-pentamethoxyflavone) (13, 14).

The third flavonoid, III, exhibited spectral characteristics similar to I and II. The 60-MHz PMR spectrum revealed four methoxy groups and signals due to four aromatic protons. The aromatic proton region of the spectrum was virtually identical (chemical shift difference <0.04 ppm) to that obtained for II. A somewhat broadened singlet at  $\delta$  5.76 (1H) could be assigned to another hydroxy group in addition to the distinctive C-5 hydroxy proton with the characteristic low-field resonance at  $\delta$  12.58.

A comparison of the mass spectra of II and III indicated a mass difference equivalent to replacing one methoxy group in II by a hydroxy group to give a molecular ion at  $m/e$  374. The characteristic fragment ions were the same as observed for the other two flavones. Based on the mass spectra and PMR data, III was concluded to be a dihydroxytetramethoxyflavone.

In the manner previously described, the UV spectra in methanol with various reagents were used to determine the positions of substituent groups (Table I). The methanol spectrum exhibited a maximum at 351 nm, well within the region expected for a flavone with a methoxy group at the 3-position, and two maxima in the 250-275-nm region, indicative of 3',4'-oxygenated flavones. Addition of sodium methoxide to the methanol solution resulted in a 34-nm bathochromic shift from 351 to 385 nm and a decrease in intensity. These results eliminated the possibility of a hydroxy group at the 4'-position (14).

The addition of sodium acetate or sodium acetate-boric acid produced no changes in the UV spectrum from that obtained in methanol alone and indicated the absence of a hydroxy group at the 3-, 7-, or 4'-position. The aluminum chloride and aluminum chloride-hydrochloric acid treatments verified, as before, a C-5 hydroxy group with an adjacent methoxy group at C-6.

The similarities in the PMR spectrum with that of artemetin and the UV spectral behavior described indicated that III was 5,3'-dihydroxy-3,6,7,4'-tetramethoxyflavone (casticin). Physical properties verified the assignment, since casticin was isolated previously from *Vitex agnus castus* (15) and was synthesized by Ho-

rhammer *et al.* (16). The isolation of these three flavones, new to *A. millefolium*, was repeated, following the same procedure, on a larger scale (4 kg of plant material) to obtain sufficient material for biological testing.

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<sup>7</sup> Varian Associates HR 220 spectrometer.