Mass Spectrometry of Polymethoxylated Flavones

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Mass spectra analyses of various polymethoxylated flavones (PMF) were obtained by gas chromatography–mass spectrometry (GC–MS). Among the compounds analyzed, mass spectra for 23 of them were recorded for the first time. In addition to common behavior of flavones under electron impact, such as retro-Diels–Alder reaction which gives a characteristic fragment ($B^{+\circ}$) from the phenyl group of the flavone skeleton, new fragmentation pathways were identified and proposed. Thus, $(B - 1)^+$, which had never been mentioned, was a characteristic ion in the spectra of the 2′-methoxyflavones; this ion resulted from the loss of a hydrogen atom from the $B^{+\circ}$ fragment. The $(M - 19)^+$ species was specific for the 3-methoxy PMF. The $(M - 15)^+$ fragment, already known in 6- and (or) 8-methoxy PMF, has been also characterized in 4′-methoxy PMF having no substituent in C-5 position.

INTRODUCTION

While flavones are widely distributed in the plant kingdom, various *Citrus* species are notable for their relatively large concentrations of polymethoxylated flavones (PMF). The highest concentrations of PMF are found in *Citrus* peel, with much lower amounts found in the juice (Tatum *et al.*, 1978; Gaydou *et al.*, 1987). PMF produce important physiological responses in the higher animals. Thus, Robbins (1977) observed that sinensetin (5,6,7,3′,4′-pentamethoxyflavone) and nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone) decrease erythrocyte aggregation and sedimentation and might be useful in the dietary control of high blood viscosity syndrome.

Recently, we reported the sensitivity and resolving power of gas capillary chromatography (GC) for PMF separation (Gaydou *et al.*, 1991). This technique was applied to the determination of PMF found in orange peel oils. Using gas chromatography–mass spectrometry (GC–MS), the flavonoid composition of honey was established without derivatization of the complex mixture (Berahia *et al.*, 1993). Thus, the use of mass spectrometry for the structural elucidation of flavones received considerable attention, but no systematic study of electron impact mass spectrometry (EIMS) of PMF was made. Retro-Diels–Alder (RDA) reaction (Figure 1) with and without hydrogen transfer, followed by the loss of CO and •OCH₃ and •CH₃ radicals (in the case of methoxylated flavonoids), is characteristic of flavonoid aglycons (Audier, 1966; Bowie and Cameron, 1966). The last known paper concerning MS of flavonoid compounds was that of Hedin and Phillips (1992). These authors recorded EIMS of various flavonoids and among them 43 flavones and flavonols. Only 6 PMF and no new mass spectra were noted in this compound family.

Since such techniques are particularly useful for natural products generally obtained in small amounts,

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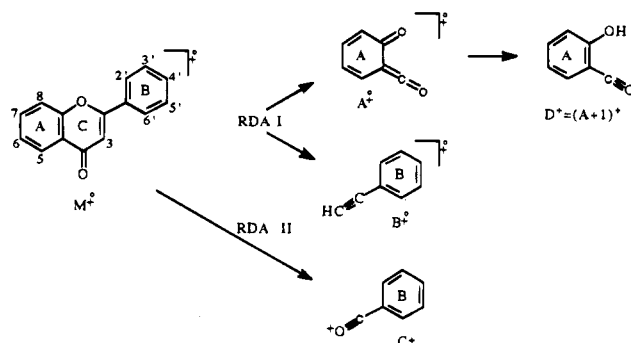

Figure 1. Flavonic skeleton with the numbering of the 10 possible substitutions and retro-Diels–Alder reactions.

we present in this paper the results obtained using GC–MS of 39 PMF substituted on rings A, B, and C as shown in Figure 1. The number of methoxy substituent ranges from 0 to 7 as shown in Table 1. We have also observed some new characteristic fragments useful in the structure determination of PMF.

MATERIALS AND METHODS

Materials. Polymethoxylated flavones were synthesized as described previously, using a modified Baker–Venkataraman procedure (Gaydou and Bianchini, 1977, 1978). Flavone **17** was obtained by methylation of apigenin (5,7,4′-trihydroxyflavone), already available in our laboratory (Gaydou and Bianchini, 1978). Products **34** and **39** were extracted using preparative chromatography of orange oil (Bianchini and Gaydou, 1981). The residue obtained, after steam distillation, was fractionated on a silica Si 60 column (E. Merck) using *n*-heptane–2-propanol as eluent. Fractions rich in PMF were recovered using preparative thin-layer chromatography (TLC) according to the method described by Tatum and Berry (1972).

GC–MS Analysis of Flavonoids. The equipment (DELSI/ NERMAG) was a gas chromatograph Di200 linked with a mass spectrometer (Automass 50 and PC-based LUCY data station). The separation of PMF was performed using a fused silica capillary column (25 m × 0.32 mm i.d.) coated with a 0.15-μm bonded phase OV-1 (A.M.L.-Chromato); the column temperature was 280 °C, and helium was used as carrier gas (inlet pressure, 0.6 bar). Electron impact mass spectrometry was carried out under the following conditions: ion source tem-

Table 1. Ion Intensities of Polymethoxylated Flavones Obtained by Gas Chromatography–Mass Spectrometry[a]

compd no.	methoxy position[b]	$M^{+\circ}$	$(M-1)^+$	$(M-15)^+$	$(M-19)^+$	$(M-28)^{+\circ}$	$(M-31)^+$	$(A+1)^+$	$A^{+\circ}$	$(B-1)^+$	$B^{+\circ}$	C^+
1		100.0	34.2			52.2		5.7	70.3		12.0	1.1
2	3	53.5	100.0	1.0	6.1		11.3	12.6	24.5			18.6
3	6	100.0	81.4			2.8	0.2	5.2	51.1		8.3	0.8
4	2′	58.5	2.1			0.6	3.9	100.0	7.9	49.3	26.2	
5	3′	100.0	17.9	1.6		27.3	12.5	2.8	18.4		38.2	
6	4′	100.0	30.4	4.8		9.1	10.3	0.1	4.5		65.2	0.6
7	5,7	100.0	55.0			5.4	19.0				5.7	6.0
8[c]	7,8	100.0	1.7	18.7					2.1		4.8	2.1
9[c]	5,4′	100.0	48.8			5.1	9.6	0.7	0.5		33.1	1.8
10[c]	6,2′	95.7	27.8				2.2	100.0	73.2	15.0	5.4	
11[c]	7,4′	100.0	28.4	1.6		10.4	2.2	1.4	9.7		43.2	0.8
12[c]	2′,3′	56.5					0.4	100.0	4.9	11.2	16.0	1.2
13[c]	2′,4′	85.2	2.2	0.5		0.6	8.1	100.0	3.8	38.9	49.8	
14[c]	2′,6′	59.5	1.0				3.2	100.0	4.6	16.8	18.7	1.1
15[c]	3′,4′	100.0	3.6	6.0			1.7	22.5	2.2		11.8	2.2
16	3,5,7	69.3	100.0	2.6	32.5		7.7	5.5	2.2			13.5
17	5,7,4′	100.0	60.1			3.8	19.1				18.6	
18[c]	7,8,4′	100.0	6.4	5.1				0.7	10.5		3.0	2.5
19[c]	5,3′,4′	100.0	40.5			2.4		1.3			4.9	2.3
20[c]	6,2′,3′	61.0	7.9					1.0	100.0	34.3	6.7	15.6
21[c]	6,2′,6′	77.9	11.4					2.8	100.0	54.4	11.3	14.5
22[c]	2′,3′,4′	100.0		1.1			2.2	68.8	1.5	11.0	29.7	
23[c]	3′,4′,5′	100.0		31.2				13.8	0.6			0.3
24	5,7,8,4′	88.0	7.7	100.0							11.0	8.3
25	3,7,3′,4′	100.0	82.7	55.0	3.8		14.3	9.9				3.9
26[c]	5,7,2′,4′	100.0	61.9			3.4	11.0			17.0	15.4	1.4
27[c]	5,2′,3′,4′	100.0	35.3			2.0	2.1	2.0	0.3	1.2	3.6	
28[c]	6,2′,3′,4′	65.3	3.9	0.2		0.6	3.0	100.0	14.7	5.4	30.0	
29[c]	5,3′,4′,5′	100.0	37.7			2.7	0.9	0.9				
30[c]	6,3′,4′,5′	100.0	9.8	23.8				0.3	11.1	6.0		5.0
31	7,3′,4′,5′	100.0	0.3	24.2					11.6			
32[c]	3,5,7,2′,4′	48.4	42.7	12.4	8.3		100.0	21.6		1.0		5.8
33	3,5,7,3′,4′	49.6	44.8	13.2	8.4		100.0	22.9		1.4		6.3
34	5,6,7,8,4′	29.2	0.6	100.0				1.6				4.2	
35[c]	5,6,7,3′,4′	13.9		100.0				22.1				3.6	3.5
36	5,7,8,3′,4′	91.2	7.9	100.0								3.6	11.7
37[c]	5,7,2′,3′,4′	100.0	41.3			1.7	13.0	1.6		0.4	3.2	0.3
38	3,5,7,8,3′,4′	100.0	79.3	82.0	22.6			24.0	2.1			30.4
39[c]	3,5,6,7,8,3′,4′	35.2	17.0	100.0	2.1			5.8				22.8

[a] Gas chromatography conditions: fused silica capillary column (25 m × 0.32 mm i.d.) coated with OV-1 (0.15-μm film thickness) at 280 °C with helium as carrier gas; mass spectra recorded at 70 eV. [b] For the determination of the methoxy position on the flavone skeleton, see Figure 1. [c] Mass spectra never cited until now.

perature, 110 °C; ionization energy, 70 eV; source pressure, 6.4 × 10⁻² mmHg; scanning from 35 to 500 amu per nominal 0.5 s.

RESULTS AND DISCUSSION

Samples are composed of the flavone 1 and five monomethoxylated (2–6), nine dimethoxylated (7–15), eight trimethoxylated (16–23), eight tetramethoxylated (24–31), six pentamethoxylated (32–37), one hexamethoxylated (38), and one heptamethoxylated (39) flavones. Although most of the PMF investigated in this work are generally found in plants, 37 compounds were obtained by synthesis (Gaydou and Bianchini, 1977, 1978). Only compounds 34 and 39 were extracted from Citrus peel oils (Bianchini and Gaydou, 1981). Among mass spectral data obtained by GC–MS of our 39 flavones listed in Table 1, 23 have never been mentioned until now.

During electron impact mass spectrometry, aglyconic flavones led to intense molecular ion $M^{+\circ}$ and other prominent fragments such as $(M-1)^+$ and $(M-15)^+$, resulting from the loss of a hydrogen or a methyl group, respectively (Nielsen and Moller, 1970; Rodriguez et al., 1972; El Gamal et al., 1986). Besides these fragmentations, two others resulting in the γ-pyrone system fragmentation, called retro-Diels–Alder I and II (RDA I and RDA II) reactions, arose as shown in Figure 1 (Audier, 1966; Kingston, 1971).
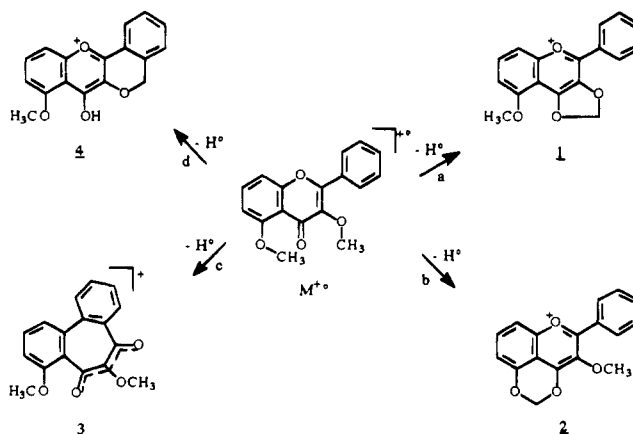
Figure 2. Different structures proposed for the $(M-1)^+$ fragment by several workers: (a, b) Kingston (1971); (c) Guidugli et al. (1984); (d) Schmid et al. (1973).

The presence of a strong $(M-1)^+$ peak was attributed to structures given in Figure 2, which could be formed after loss of a hydrogen atom from the 3- or 5-methoxy group to give ions 1 and 2, respectively (Kingston, 1971) or after loss of an aromatic hydrogen from the 2′-position to give ions 3 and 4 (Guidugli et al., 1984; Schmid et al., 1973).

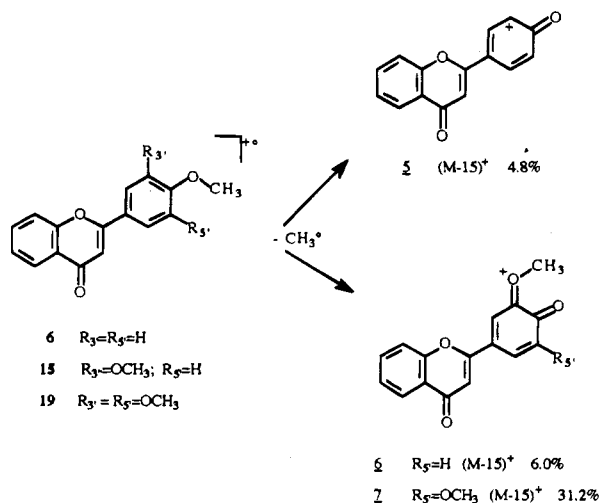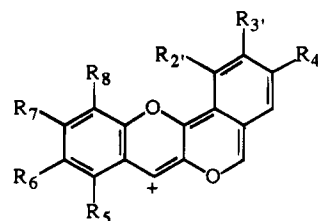In agreement with previous works (Kingston, 1971; Rizzi and Boeing, 1984), the 6- and 8-methoxyflavones

Figure 3. Formation of $(M - 15)^+$ ion from 4'-methoxyflavones.

Compound N°	R5	R6	R7	R8	R2'	R3'	R4'
2	H	H	H	H	H	H	H
16	OCH₃	H	OCH₃	H	H	H	H
25	H	H	OCH₃	H	H	OCH₃	OCH₃
32	OCH₃	H	OCH₃	H	OCH₃	H	OCH₃
33	OCH₃	H	OCH₃	H	H	OCH₃	OCH₃
38	OCH₃	H	OCH₃	OCH₃	H	OCH₃	OCH₃
39	OCH₃	OCH₃	OCH₃	OCH₃	H	OCH₃	OCH₃

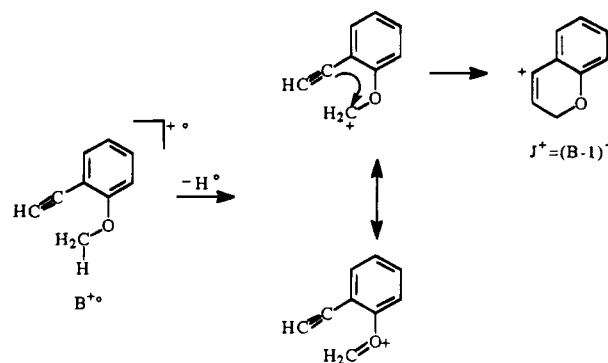**Figure 4.** Structure proposed for the $(M - 19)^+$ ion species observed in the case of various 3-methoxyflavones.

Figure 5. Formation of $(B - 1)^+$ ion from $B^{+°}$ fragment.

with more than two substituents on the A-ring showed intense elimination of the methyl group because of stable quinonoid ion formations $(M - 15)^+$. This ion constituted the base peak in such compounds as **24, 34–36**, and **39** except in the case of compound **38**. The $M^{+°}$ intensity value for the 8-methoxy PMF was very high (from 88 to 100%). Goudard *et al.* (1979) observed that the $M^{+°}$ ions were the base peaks in 5,7,8-trimethoxy PMF spectra. We proposed another feature which afforded the formation of an analogous fragment. It concerned PMF which possessed a 4'-methoxy function and no substitution in the 5-position (Figure 3). The $(M - 15)^+$ fragment that was absent for 5,3',4',5'-tetramethoxyflavone appeared for 6,3',4',5'-tetramethoxyflavone (23.8%) and 7,3',4',5'-tetramethoxyflavone (24.2%). Furthermore, the $(M - 15)^+$ process was in relation with the 3'- or (and) 5'-substitution(s). Thus, concerning the stability and, hence, the number of resonance structures, the $(M - 15)^+$ ion was of lower intensity in fragment 5 of 4'-methoxyflavone (**6**) (4.8%) than in fragments 6 and 7 of 3',4'-dimethoxyflavone (**15**) and 3',4',5'-trimethoxyflavone (**19**) (6 and 31.2%, respectively) (Figure 3).

A $(M - 19)^+$ ion was observed in the case of seven PMF: **2, 16, 25, 32, 33, 38**, and **39**. A $(M - 19)^+$ peak was previously described by Kingston (1971) in the case of five 3,5-dimethoxyflavones. As shown in Table 1, all of these compounds have a methoxy group in the 3-position (3-methylflavonol). Therefore, we propose a tetracyclic structure (Figure 4) for this $(M - 19)^+$ ion, obtained by the loss of 19 amu.

The MS data exhibited $(A - 15)^+$ and $(A - 43)^+$ ions in accordance with flavone having a methoxy group at C-6 or C-8. $(A - 15)^+$ was formed from $(M - 15)^+$ by the retro-Diels–Alder reaction; the departure of 28 amu corresponding to one carbonyl group yielded $(A - 43)^+$ (Kingston, 1971). The $(A - 15)^+$ and $(A - 43)^+$ fragments were more intense for the 8-methoxylated flavones than for the 6-methoxyflavones. This result could be explained by the greater stability of p-quinonoid over o-quinonoid ions (Barberan *et al.*, 1986).

In the GC–MS analysis of PMF investigated, a strong $(A + 1)^+$ peak was observed; it constituted the base peak for the 2'-methoxy PMF unsubstituted in the A-ring and 6-monosubstituted in the same ring (fragment D^+, Figure 1). Only one exception was observed, 2',3',4'-trimethoxyflavone (68.8%, Table 1). In the case of 2'-methoxy PMF substituted in C-5 position, the intensity

value for the D^+ ion was weak or absent since for this last position the $(M - 1)^+$ fragment occurred preferably. For Audier (1966), D^+ presumably arose by a hydrogen atom transfer during RDA I (Figure 1).

Another fragment mentioned here, for the first time, was found to be characteristic for the 2'-methoxyflavones. We proposed for the $(B - 1)^+$ ion a bicyclic structure type Δ^3-chromene (Figure 5). This species could be obtained from $B^{+°}$ by an intramolecular process. First, a carbocation was formed by loss of a hydrogen radical; in a second step, the electrophilic carbon was attacked by the triple bond rich in electrons; finally, the alcenyl cation $(B - H)^+$ was afforded (Figure 5). We named this ion J^+ by referring to Audier's convention and in continuation of his symbolism (Audier, 1966).

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