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Gas Chromatography and Mass Spectrometry of Methylated Xanthines[1,2]

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Gas chromatography and mass spectrometry are described for the analysis of methylated xanthines and their derivatives. A satisfactory separation of these compounds was achieved using 2% OV-17 and 3% XE-60 columns. Molecular ions of the methylated xanthines were observed as abundant peaks, and the trifluoroacetyl derivatives especially appear with base peaks. The fragmentation mechanisms were discussed.

In the preceding paper, the authors reported a method of detection and identification of urine-extracted phenylpyrazolone derivatives by combined gas chromatography and mass spectrometry (GC–MS).[1a]

The present paper describes the analysis of methylated xanthines, which are therapeutic agents using gas chromatography and mass spectrometry. Those are, for example, caffeine (Ca), theobromine (Tb), theophylline (Tp), oxyethyltheophylline (Oe), oxypropyltheophylline (Op), and dyphylline (Dp). This study attempts to get basic informations for their identification by means of the GC–MS, and is a part of the systematic study of the doping test for race horses.

Experimental[4]

Materials and Reagents——The methylated xanthines were purchased from the following commercial sources; Ca from Wako Pure Chemical Industries, Ltd., Tb and Tp from Tokyo Kasei Kogyo Co., Ltd., Oe from Sankyo Co., Ltd., Op from Takeda Chemical Industries, Ltd., and Dp from Iwaki Seiyaku Co., Ltd.

Apparatus——Gas chromatography was performed on a Hitachi K-53 gas chromatograph equipped with a hydrogen flame ionization detector. The stainless steel tubes (1 m×3 mm i.d. or 2 m×3 mm i.d.) were silanized with hexamethyldisilazane and trimethylchlorosilane, and were packed with Chromosorb

TABLE I. TMS Derivatives of Oe, Op, and Dp

Compd.	mp (°C)	Yield (%)		Analysis (%) Calcd. C	H	N	Found C	H	N
Oe	78—79	75	$C_{12}H_{20}O_3N_4Si$	48.62	6.80	18.90	48.84	6.90	19.09
Op	72—74	81	$C_{13}H_{22}O_3N_4Si$	50.30	7.14	18.05	50.34	7.21	18.13
Dp	73—74	65	$C_{16}H_{30}O_4N_4Si$	48.21	7.59	14.06	48.23	7.64	14.10

1) *a*) This paper constitutes Part III of the series entitled "Studies on Doping Test by Gas Chromatography-Mass Spectrometry." Part II: A. Momose and T. Tsuji, *Yakugaku Zasshi*, **92**, 193 (1972); *b*) This study was presented in part in the 89th Annual Meeting of the Pharmaceutical Society of Japan, Nagoya, April 1969.

2) In this paper the following common names are used: caffeine=1,3,7-trimethylxanthine, theobromine=3,7-dimethylxanthine, theophylline=1,3-dimethylxanthine, oxyethyltheophylline=7-(2-hydroxyethyl)-1,3-dimethylxanthine, oxypropyltheophylline=7-(2-hydroxypropyl)-1,3-dimethylxanthine, and dyphylline=7-(2,3-dihydroxypropyl)-1,3-dimethylxanthine.

3) Location: *Kamiyoga, Setagaya-ku, Tokyo.*

4) All melting points are uncorrected.

W. AW. DMCS (80—100 mesh) coated by 1.5% SE-30, 3% SE-30, 1.5% SE-52, 3% QF-1, 2% OV-17, and 3% XE-60 respectively. Mass spectrometry was performed on a Hitachi RMU-7E double focusing mass spectrometer, and the high resolution mass spectrum was measured by means of an on-line Hitachi 002-type high resolution mass system. The ionizing voltage was maintained at 70 eV.

Preparation of Trimethylsilyl (TMS) Derivatives——One gram portions of the samples were dissolved in 20 ml of anhydrous pyridine and trimethylsilylated with 5 ml of N,O-bis(trimethylsilyl)acetamide at 40—50° for 30—40 min in a water bath. Then, each reaction mixture was evaporated *in vacuo*, and the residual syrup was crystallized by letting stand overnight at room temperature, and recrystallized from ether and petroleum ether to give colorless needles. These are shown in Table I.

Preparation of Trifluoroacetyl (TFA) Derivatives——Five hundred mg of sample was dissolved in 4 ml of tetrahydrofuran, 2 ml of trifluoroacetyl anhydride was added, and maintained for 10 min at 70° in a water bath. After filtration, each reaction mixture was chilled in an ice bath and separated precipitate was recrystallized from ether to give colorless needles. These are shown in Table II.

Table II. TFA Derivatives of Oe, Op, and Dp

Compd.	mp (°C)	Yield (%)	Formula	Analysis (%) Calcd. C	H	N	Found C	H	N
Oe	180—181	67	$C_{11}H_{11}O_4N_4F_3$	41.26	3.46	17.50	41.30	3.72	17.45
Op	160—162	63	$C_{12}H_{13}O_4N_4F_3$	43.12	3.92	16.76	42.99	3.93	16.58
Dp	170—171	71	$C_{14}H_{12}O_6N_4F_6$	37.68	2.71	12.56	37.87	2.90	12.65

Result and Discussion

Gas Chromatography

The gas chromatographic separation of caffeine has been reported by the use of various phases,[5] but only a few reports have been described to the other methylated xanthines.[6,7] Moreover, these methods were not entirely satisfactory, mainly because of their poor sensitivity or the experimental conditions.

An initial attempt was made to establish the appropriate column conditions for the separation of six methylated xanthines. The retention values of these compounds under a variety of conditions are listed in Table III. A good separation was obtained on 2% OV-17 and 3% XE-60 columns. A typical gas chromatogram of the methylated xanthines and an internal reference is shown Fig. 1. The lowest limits of detection of these compounds were about 0.1—3.0 μg.

Table III. Relative Retention Times for Methylated Xanthines[a]

Compd.	1.5% SE-30 190°	3% SE-30 200°	1.5% SE-52 210°	3% QF-1 210°	2% OV-1 205°	2% OV-17 240°	3% XE-60 230°
Ca	0.43	0.42	0.45	0.46	0.49	0.45	0.32
Tb	0.61	0.82	0.61	0.77	0.77	0.54	0.57
Tp	1.02	1.00	0.95	0.85	0.80	0.81	1.19
Op	1.00 (4.1min)	1.00 (3.3min)	1.00 (4.4min)	1.00 (3.9min)	1.00 (3.5min)	1.00 (6.3min)	1.00 (3.7min)
Oe	1.15	1.03	1.16	1.18	1.29	1.19	1.38
Dp	3.29	3.82	2.75	2.51	3.23	2.49	3.78

a) column: 2 m×3 mm, stainless steel, treated with HMDS. the flow rate of nitrogen 60 ml/min

5) E.G.C. Clark, "Isolation and Identification of Drugs," The Pharmaceutical Press, London, 1969, p. 234.
6) K.D. Parker, C.R. Fontan, and P.L. Kirk, *Anal. Chem.*, 35, 356 (1963).
7) J. Reisch and H. Walker, *Pharmazie*, 21, 467 (1966).

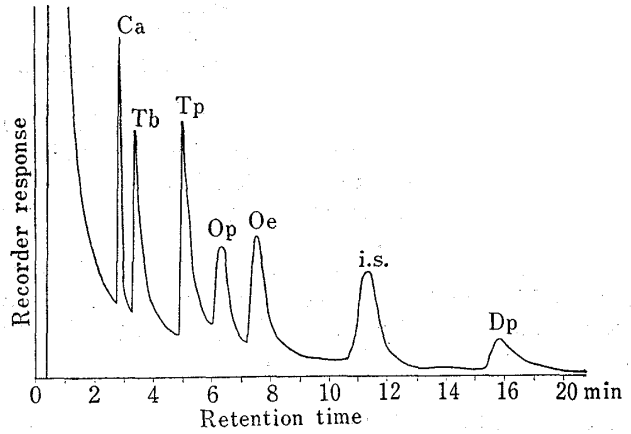

Fig. 1. Gas Chromatogram of the Methylated Xanthines with OV-17 Column

conditions: column, 2% OV-17 (2 m×3 mm) stainless steel, treated with HMDS. temperature[a]; Tc 240°, Ti 270°. carrier; nitrogen 60 ml/min internal standard(i.s.); dioctylphthalate
a) The following abbreviations were used in this paper. Tc: column temperature, Ti: injection port temperature

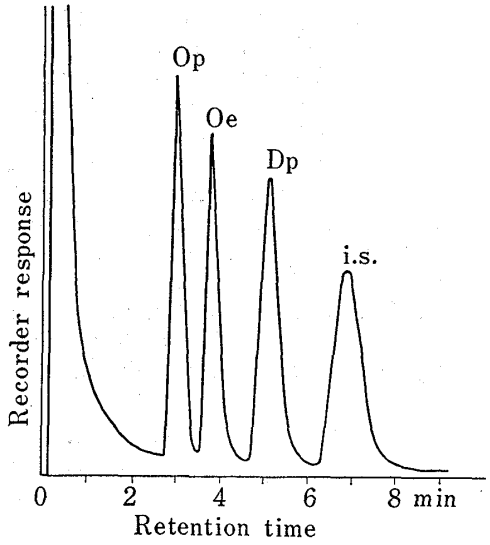

Fig. 2. Gas Chromatogram of TMS Derivatives

conditions: column; 3% XE-60 (1 m×3 mm) stainless steel, treated with HMDS. Tc 210°, Ti 230°, nitrogen; 60 ml/min internal standard (i.s.) dioctylphthalate

In the case of theophylline derivatives, the preparation of TMS[8] and TFA derivatives[9] is effective in achieving a more sensitive examination. These TMS and TFA derivatives were obtained as stable crystalline compounds (see Experimental section). Typical gas chromatograms of the TMS and TFA derivatives on the 3% XE-60 column are shown in Fig. 2 and Fig. 3 respectively. The calibration curves for each TFA derivative are shown in Fig. 4, showing a good linearity. The lowest limits of detection obtained were about 1/10—1/20 than those of the original compounds.

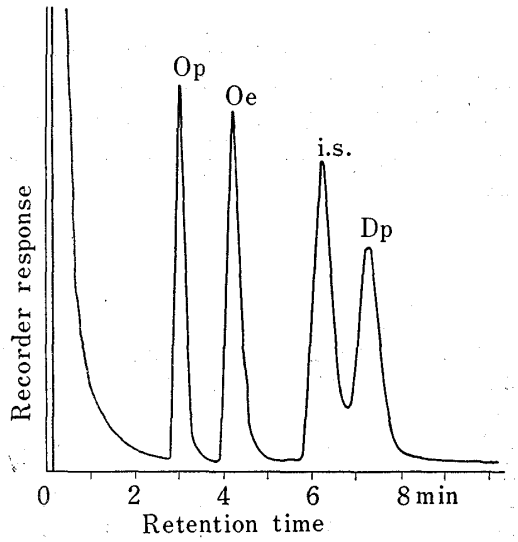

Fig. 3. Gas Chromatogram of TFA Derivatives

conditions: column; 3% XE-60 (1 m×3 mm), Tc 220°, Ti 235°, nitrogen 60 ml/min internal standard (i.s.) dicyclohexylphthalate

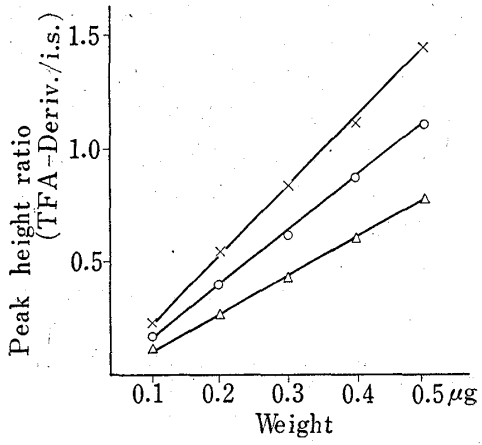

Fig. 4. Calibration Curves for TFA Derivatives using Dicyclohexylphthalate as an Internal Standard

conditions: 3% XE-60 (1 m×3 mm) stainless steel
Tc Op and Oe-TFA at 215°, Dp at 200°
Ti 230°, nitrogen 60 ml/min attenuation ×20

8) J.F. Klebe, H. Finkbeiner, and D.M. White, *J. Am. Chem. Soc.*, **88**, 3390 (1966).
9) T. Imanari, Y. Arakawa, and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **17**, 1967 (1969).

Mass Spectrometry

The mass spectrometry of methylated xanthine has been reported by Spiteller and Spiteller-Friedmann.[10] However, the theophylline derivatives have not been described. In this section the authors mainly describe the mass spectrometry of Oe, Op, and Dp and its derivatives. The mass spectra of the methylated xanthines are shown in Fig. 5. The molecular ion peaks of their compounds were observed as abundant peaks. In the spectra of theophylline derivatives, the corresponding M-H_2O ions were observed at *m/e* 206, 220, and 236 respectively. In the spectrum of the Dp, the ion at *m/e* 224 would be afforded from the molecular ion through the β-fission of the aliphatic alcohol group.

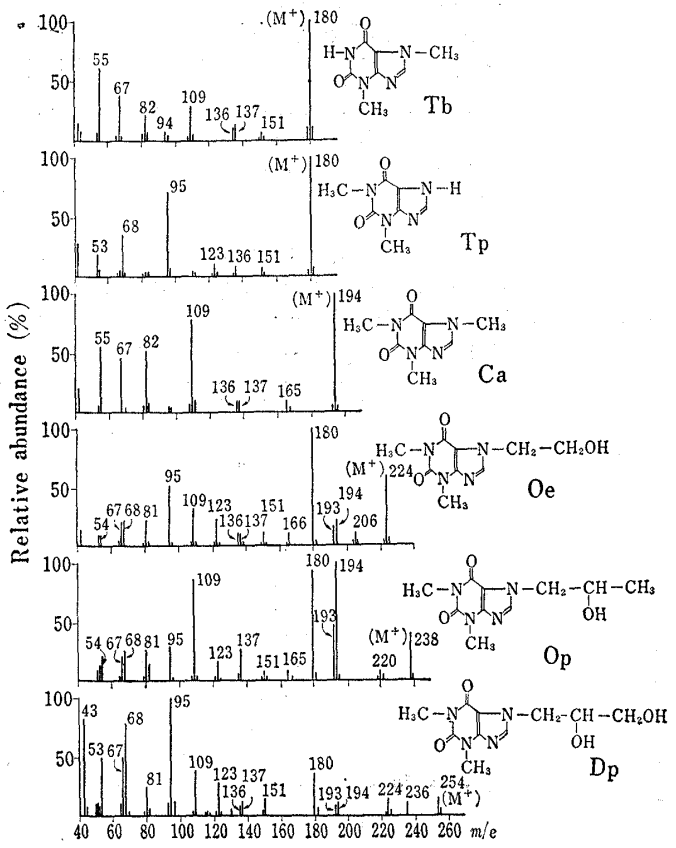

Fig. 5. Mass Spectra of Methylated Xanthines

The fragment ions at *m/e* 194 and 180 in the spectra of theophylline derivatives were found to correspond to $C_8H_{10}O_2N_4$ and $C_7H_8O_2N_4$ by high resolution mass measurements (HRMS). These ions may be represented as ionized Ca and Tp, which are formed directly from the molecular ions. These fragmentation processes are supported by the presence of the corresponding metastable ions (Chart 1). The *m/e* 194 and 180 fragment ions could undergo further break-up which resembled the decomposition of Ca and Tp[10] respectively.

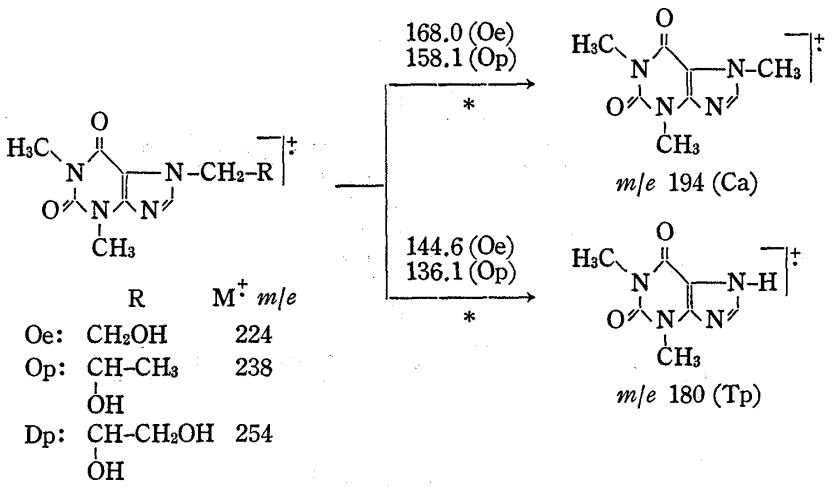

Chart 1

10) G. Spiteller and M. Spiteller-Friedmann, *Monatsh.*, **93**, 632 (1962).

The mass spectra of TMS derivatives of Oe, Op, and Dp (I—III) are shown in Fig. 6. The molecular ions and M-15 ion **a** of these compounds were observed as abundant peaks. An ion at *m/e* 252 **b** in the spectra of their derivatives was found to correspond to $C_{10}H_{16}O_2N_4Si$ by HRMS. For the formation of this ion, an one-step decomposition of molecular ions can be postulated, and it was supported by the presence of the appropriate metastable ions at *m/e* 214.5 for I, 204.9 for II and 159.5 for III. The subsequent decomposition of **b** by the expulsion of the methyl radical afforded the ion at *m/e* 237 **c**, which was confirmed to be $C_9H_{13}O_2N_4Si$ by HRMS. In the case of III, the fragmentation process to form this ion was supported by the observation of a metastable ion at *m/e* 222.9. In the cases of I and II, another process to form the **c** ion from **a** can be postulated. This assumption was supported by the presence of the appropriate metastable ions at *m/e* 199.9 and 190.4 for **a** respectively (Chart 2).

Djerassi, *et al.*[11] found that certain trimethylsilyl ethers undergo skeletal rearrangement upon electron impact by studying the mass spectrum of benzyl trimethylsilyl ether.

The fragment ion at *m/e* 266 **d** in the spectra of TMS derivatives was found by HRMS to correspond to $C_{11}H_{18}O_2N_4Si$. In the spectra of I and II, there are metastable ions at *m/e* 239.0 (M→**d**) and 228.2 (M→**d**) respectively. Thus it may be considered that they are formed directly from the molecular ions by loss of HCHO and CH_3CHO. However, in the case of III, the **d** ion may be produced through another course, where the molecular ion may eliminate $(CH_3)_3Si-OH$ to give the *m/e* 308 ion ($C_{11}H_{28}O_4N_4Si$: measured 308.184, calcd. 308.187 by HRMS), following the loss of C_2H_2O. This fragmentation process was documented by the presence of metastable ions at *m/e* 238.4 (M→308) and 228.0 (308→**d**) in the spectrum, and by the absence of an appropriate metastable ion for the direct formation of **d** from the molecular ion. The **d** ion further decomposed by the loss of hydrogen atom to afford the *m/e* 265 **e** ion.

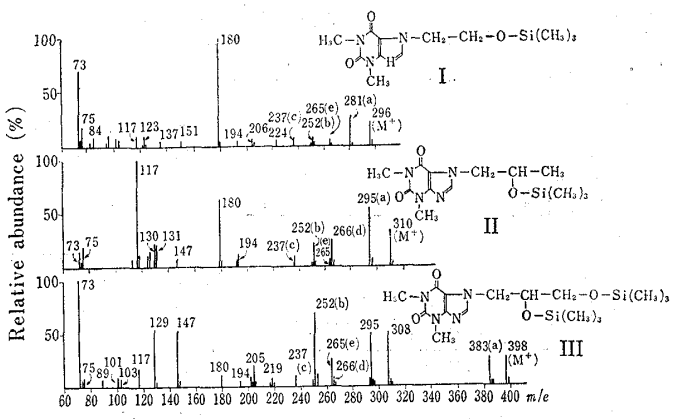

Fig. 6. Mass Spectra of TMS Derivatives

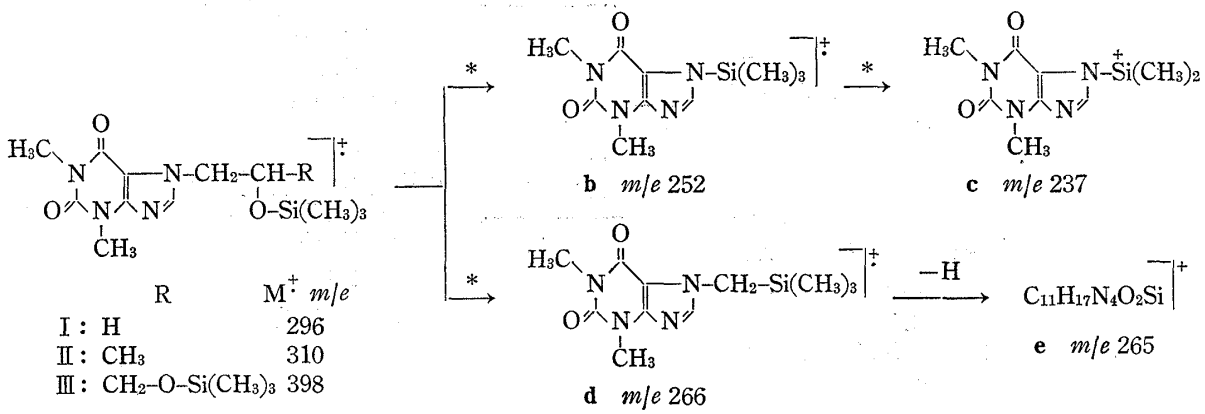

Chart 2

11) J. Diekman, J.B. Thomson, and C. Djerassi, *J. Org. Chem.* 32, 3904 (1967).

Peaks corresponding to the ionized Ca (*m/e* 194) and Tp (*m/e* 180) also occurred in the spectra of TMS derivatives, and the intensity of these ions varied from 5—100%. The fragment ion at *m/e* 117 in the spectra of I and II corresponds to $C_5H_{13}OSi$ by HRMS, and in the case of II, this ion is observed as a base peak. Thus, it may be considered that the α-fission of trimethylsilyl ether of secondary alcohol occurs.[12] In the spectrum of III, however, the *m/e* 117 fragment ion corresponds to $C_4H_9O_2Si$ as found by HRMS, and may be assigned the structure of $H\text{-}\overset{\overset{O}{\|}}{C}\text{-}CH_2O\text{=}Si(CH_3)_2$.

The mass spectra of the TFA derivatives of Oe, Op, and Dp (IV—VI) are shown in Fig. 7. The molecular ions of these compounds are observed as base peaks. Many fragments can be explained through the elimination of CF_3CO (**a**), CF_3COO (**b**), and CF_3COOH (**e**), and the α- (**c**) and β- (**d**) cleavages of the primary or secondary alcohols are also observed. These fragmentation processes were supported by the presence of the corresponding metastable ions. The fragmentation process of **e** from the molecular ion may be considered to be an example of the McLafferty rearrangement.[13] The fragment ion at *m/e* 219 in the spectrum of VI was found to correspond to $C_{10}H_{10}O_2N_4$ by HRMS, and may be represented as a product by the elimination of trifluoroacetic acid from **b**.

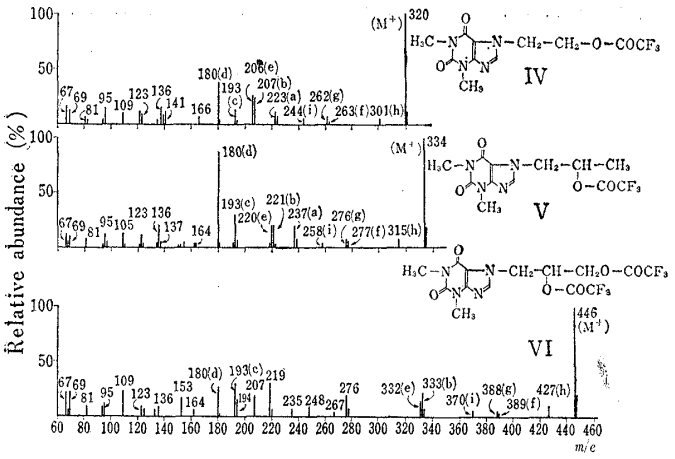

Fig. 7. Mass Spectra of TFA Derivatives

The mass spectra of all the TFA derivatives examined also contain weak peaks corresponding to the loss of the fluorine ion **h**. These $(M\text{-}F)^+$ ions produce an **i** ion by the elimination of methylisocyanate due to the retro-Diels-Alder decomposition.[14] On the other hand, the first step in the fragmentation of TFA derivatives seems to be the retro-Diels-Alder decomposition and the production of the **f** ion, which would loss successively a hydrogen atom to give **g** ion. The processes of these

Table IV. Corresponding Peaks in Mass Spectra of TFA Derivatives

Ions	IV	(compn.)[a]	V	(compn.)[a]	VI	(compn.)[a]
a	223	($C_9H_{11}O_3N_4$)	237	($C_{10}H_{13}O_3N_4$)	—	
b	207	($C_9H_{11}O_2N_4$)	221	($C_{10}H_{13}O_2N_4$)	333	($C_{10}H_{12}O_4N_4F_3$)
c	193	($C_8H_9O_2N_4$)	193	($C_8H_9O_2N_4$)	193	($C_8H_9O_2N_4$)
d	180	($C_7H_8O_2N_4$)	180	($C_7H_8O_2N_4$)	180	($C_7H_8O_2N_4$)
e	206	($C_9H_{10}O_2N_4$)	220	($C_{10}H_{12}O_2N_4$)	332	($C_{10}H_{11}O_4N_4F_3$)
f	263	($C_9H_8O_3N_3F_3$)	277	($C_{10}H_{10}O_3N_3F_3$)	389	($C_{12}H_9O_5N_3F_6$)
g	262	($C_9H_7O_3N_3F_3$)	276	($C_{10}H_9O_3N_3F_3$)	388	($C_{12}H_8O_5N_3F_6$)
h	301	($C_{11}H_{11}O_4N_4F_2$)	315	($C_{12}H_{13}O_4N_4F_2$)	427	($C_{14}H_{12}O_6N_4F_5$)
i	244	($C_9H_8O_3N_3F_2$)	258	($C_{10}H_{10}O_3N_3F_2$)	370	($C_{12}H_9O_5N_3F_5$)

a) Composition of each fragment was determined by high resolution mass spectrometer (HITACHI RMU-7E).

12) G.J. Karabatsos, R.A. Mount, D.O. Rickter, and S. Meyerson, *J. Am. Chem. Soc.*, **88**, 5651 (1966).
13) *a*) W. Benz and K. Biemann, *J. Am. Chem. Soc.*, **86**, 2375 (1964); *b*) O.S. Chizhov, B.A. Dmitriev, B.M. Zolotarev, A. Ya. Chernyak, and N.K. Kochetkov, *Org. Mass Spectrom.*, **2**, 947 (1969).
14) J.M. Rice, G.O. Dudek, and M. Barber, *J. Am. Chem. Soc.*, **87**, 4569 (1965).

decomposition were supported by the presence of the corresponding metastable ions or HRMS (Table IV).

The fragment ion at *m/e* 180 (ionized Tp) in the spectra of TFA derivatives could undergo further break-up which resembled the Tp decomposition.

Conclusion

We have achieved a good separation of methylated xanthines on 2% OV-17 and 3% XE-60 columns, and in the case of theophylline derivatives, a good sensitivity was achieved as TMS and TFA derivatives on a 3% XE-60 column.

The mass spectrometric analysis of the methylated xanthines showed excellent properties. The molecular ion was, in each case, an abundant peak, which gave usually the intensity of over 15%. On the other hand, Ca, Tb, and Tp showed the base peak. Furthermore, the molecular ions in the spectra of TFA derivatives gave the base peak.

The above results suggest that GC–MS may well be applied to the identification of methylated xanthines.

Acknowledgement The authors wish to thank Mr. M. Murata for his technical assistance.