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Study on the Components of Musk. I.[1] Ether Soluble Components

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Ether soluble components of musk have been investigated by chromatographic and spectroscopic methods. As indicated in the list of identified constituents (Table II), they were proved to be predominantly steroidal: eleven androstane derivatives, cholesterol and its esters, and cholest-4-en-3-one, in addition to wax and muscone. Characteristic feature of cholesterol esters and wax was that they are the esters derived from very long chain fatty acids of C_{14} through C_{40} in which branched ones were predominant except C_{16} and C_{18}. Wax alcohols were found to have almost exclusively branched chain (C_{20} to C_{34}).

The musk is a substance secreted in gland by male musk deer (*Moscus moschiferus* Linne). Aside from the well known use as the basis of numerous perfumes, it is one of the most important chinese drugs as is listed in Japanese Pharmacopea Part II. Pharmacological studies have indicated that the musk exhibits cardiovascular stimulating,[3] male hormonal[4] and antiinflammatory activities,[5] and that induces the potentiation of β-adrenergic effect.[6] In spite of being relatively expensive traditional drug possessing such characteristic pharmacological activities, no systematic investigation on the chemical components has been reported, only muscone[7] and muscopyridine[8] were isolated and characterized by Ruzicka, *et al.*

This paper describes the isolation and identification of ether soluble components of musk by means of principal use of chromatographic and spectroscopic methods.

Experimental

Material——The ether extract of musk (Nepal product) was kindly provided by professors M. Kimura and I. Waki of this university. It was obtained in approximately 10% yield after extraction of bag content by a Soxhlet apparatus. Authentic samples were purchased or synthesized according to the published methods to which were given references.

Chromatographic Procedures——Thin-layer Chromatography (TLC): Analytical thin-layer chromatographic plates used were 20×20 cm precoated abrasion resistant silica gel GF_{254} (Merck AG, 0.25 mm). Thick-layer for preparative scale was prepared by coating water slurry of 20 g of silica gel $PF_{254}{}^{+}{}_{366}$ (Merck AG) on 20×20 cm glass plate, air drying, and activation by heating at 120° for 1 hr. Developing solvent systems were: I, benzene; II, benzene–ether (10+90); III, benzene–ethanol (90+10); IV, benzene–ethyl acetat (50+50). Spots on analytical plate were detected by iodine vapor (for lipid), or by spraying 60% sulfuric acid or alcoholic vanillin-concentrated sulfuric acid reagent[9] followed by heating at 115—120° in an oven (for steroids). In the case of preparative scale procedure bands were made visible by longwave ultraviolet light (366 nm), scraped off, and extracted with ether by a Soxhlet apparatus.

Gas Chromatography (GC): Isothermal GC was performed by JEOL JGC-20 KF equipped with flame ionization detector and glass spiral column of 1 m×2 mm i.d. using helium as carrier gas (inlet pressure

1) This research formed part of the PhD. Thesis presented by J.C. Do to Yeung Nam University, Taegu, Korea, from which he was on leave.
2) Location: *Gofuku, Toyama.*
3) Y. Takayama, *J. Exp. Med.*, **15**, 435 (1930).
4) T. Sano, *Yakugaku Zasshi*, **56**, 913 (1936); *ibid.*, **57**, 851 (1937).
5) R.K. Mishra, R.B. Arora, and S.D. Seth, *J. Pharm. Pharmacol.*, **14**, 830 (1962).
6) *a*) M. Kimura and I. Waki, *Japan J. Pharmacol.*, **16**, 129 (1966); *b*) M. Kimura, I. Waki, and H. Ikeda, *Yakugaku Zasshi*, **88**, 130 (1968).
7) L. Ruzicka, *Helv. Chim. Acta*, **9**, 715, 1008 (1926).
8) H. Schinz, L. Ruzicka, U. Geyer, and V. Prelog, *Helv. Chim. Acta*, **29**, 1524 (1946).
9) B.P. Lisboa, *J. Chromatog.*, **19**, 81 (1965).

0.6 kg/cm²). The column packings and operating temperatures for steroid analyses were (retention times of 5α-cholestane were indicated in parenthesis): (I) OV-17 1.5% on dimethyldichlorosilane treated Gas-Chrom Q 80/100 mesh at 210° (15.8 min) and 235° (6.3 min); (II) QF-1 1.5% on dimethyldichlorosilane treated Chromosorb W 80/100 mesh at 193° (10.9 min). NGS column (1.5% on acid-washed Chromosorb W 60/80 mesh) was also employed for the analyses of fatty alcohols and fatty acid esters. Programmed GC was carried out by Shimazu GC-4BPF with flame ionization attachment. In this case the glass column of 2 m × 4 mm i.d. was packed with OV-17 1% on silanized Gas-Chrom Q 80/100 mesh and nitrogen was used as carrier gas (inlet pressure 0.8 kg/cm²). Temperature program: 5°/min.

Spectrum Determination——Infrared spectra (IR) and ultraviolet spectra (UV) were recorded by Nippon Bunko IR-S and Hitachi 124 spectrophotometers, respectively. The mass spectra were obtained with JEOL JMS-01SG-2 instrument under the following conditions: ion source temperature 280°, ionizing energy 75 eV, ionizing current 200 μA. For GC-MS procedure the JGC-20KF Gas Chromatograph was coupled, *via* JMS enricher maintained at 240°, to the source of mass spectrometer.

Chemical Reactions——Trimethylsilation for GC of hydroxy compounds was carried out by dissolving a sample in bistrimethylsilylacetamide (when solubility required, pyridine was added) and standing overnight at room temperature or heating at 60—70° for 1 hr. The solution was injected directly. Saponification of ester components was carried out by refluxing with 10% methanolic KOH for 10 to 20 hr. Neutral and acid fractions were obtained by usual manner. Acid fraction was then esterified with BF_3–MeOH and purified by preparative TLC. Chromic acid oxidation of hydroxy steroids was done by Jones reagent[10] using acetone as solvent.

Result and Discussion

Thin-layer chromatogram of musk ether extract is shown in Fig. 1. The spots were separated by preparative scale procedure as illustrated in Fig. 2: first with solvent system I yielding seven fractions, and then the most polar fraction VII with solvent system III into six fractions. The yield of each fraction is given in Table I.

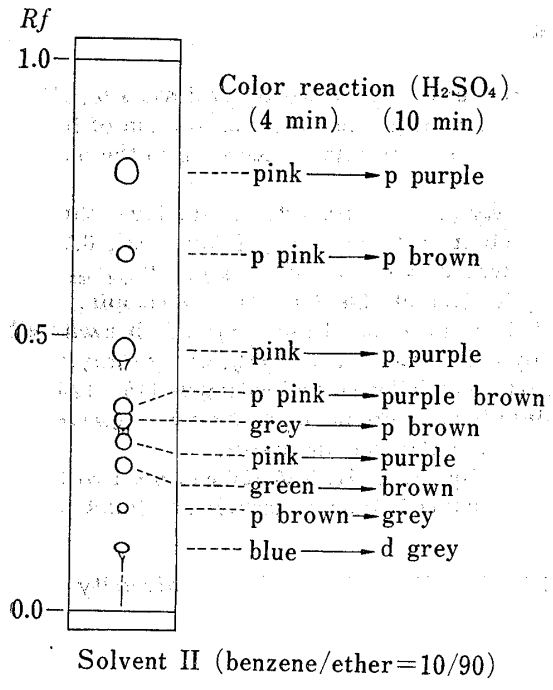

Fig. 1. Thin-layer Chromatogram of Musk Ether Extract
abbreviation: p=pale, d=dark

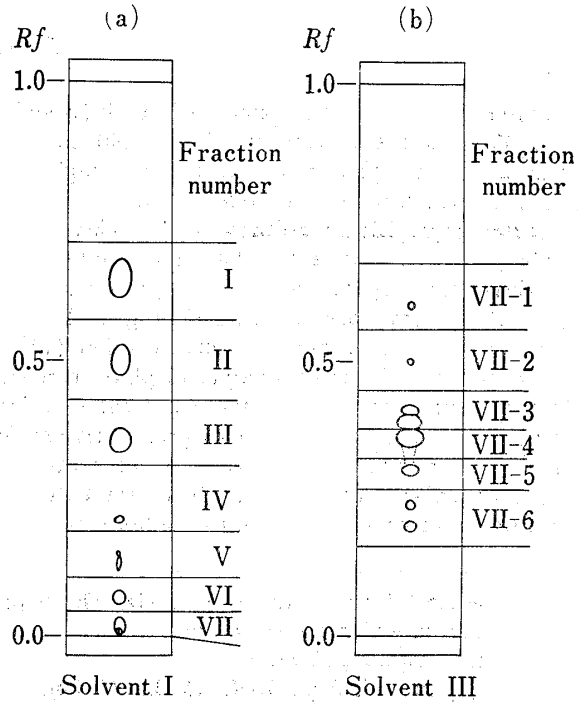

Fig. 2. Thin-layer Chromatogram for the Fractionation of Musk Ether Extract
(a) First fractionation with benzene (b) Fractionation of VII obtained in (a) with benzene/ethanol=90/10

10) R.S. Monson, "Advanced Organic Synthesis," Academic Press, New York, 1971, p. 1.

TABLE I. Preparative Thick–Layer Chromatography of Musk Ether Extract

Fraction[a)]	Weight (mg)	Fraction[b)]	Weight (mg)
I	60	VII-1	9
II	20	VII-2	12
III	113	VII-3	15
IV	14	VII-4	25
V	15	VII-5	14
VI	25	VII-6	32
VII	232		

a) 500 mg of ether extract was used with solvent I.
b) 165 mg of Fraction VII was used with solvent III.

Characterization of fractionated components thus obtained are described below in the order of fraction numbers.

Fraction I

Since infrared spectrum showed an ester $\nu_{C=O}$ band at 1735 cm^{-1}, this fraction was saponified. Neutral substances obtained were revealed by MS to be a mixture of cholesterol and small amount of cholestanol. Methyl esters derived from carboxylic acid fraction showed a complex GC pattern as shown in Fig. 3. The range of chain length was determined to be C_{14}

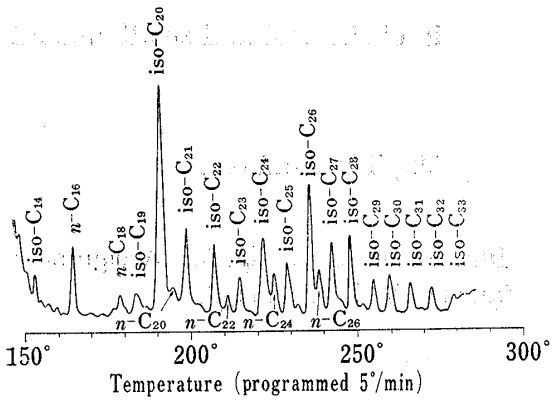

Fig. 3. Gas Chromatogram of Methylesters obtained from Fraction I (Cholesterol Esters)

OV-17 column, condition as described under experimental section. iso-C_n means branched chain ester of carbon number *n*. *n*-C_n menas straight chain ester of carbon number *n*.

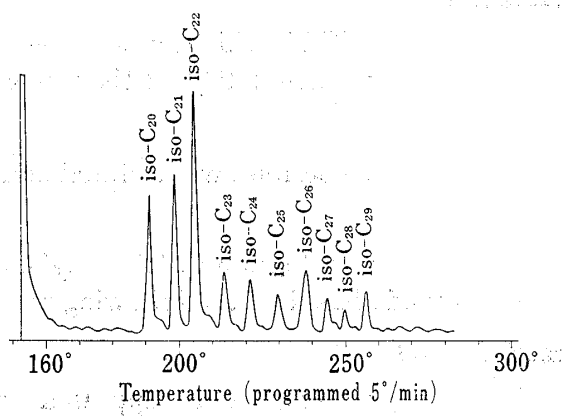

Fig. 4. Gas Chromatogram of Alcohols obtained from Fraction II (Wax)

OV-17 column, condition as discribed under experimental section. iso-C_n means branched chain alcohol of carbon number *n*.

through C_{40} by measurement of MS at various temperatures, and peak assignment indicated in the gas chromatogram was made by GC-MS (between C_{20} and C_{26}) and by employing a linear relationship between the logarithm of retention times and chain length. It is interesting that the branched chain saturated esters are present in significantly greater quantity except C_{16} and C_{18}, and that unsaturated counterparts could not be detected by GC-MS. The structures of branched chain esters were not determined in the present investigation. but MS indicated that they are branched at positions higher than ten (presumably so called iso and anteiso structures)[11] since there were observed the characteristic peaks of normal chain (*m/e* 74, 87, 143, 199) with expected variations of peak heights.[12]

11) N. Pelick and J.W. Shigley, *J. Am. Oil Chemists' Soc.*, **44**, 121 (1967).
12) R. Ryhage and E. Stenhagen, *Arkiv Kemi*, **15**, 291 (1960); *J. Lipid Res.*, **1**, 361 (1960).

Fraction II

This fraction should also contain an ester component showing $\nu_{C=O}$ at 1740 cm^{-1}, and therefore subjected to saponification. Methyl ester mixture obtained from fatty acid fraction had almost the same composition as that of the previous fraction with respect to chain length distribution and normal-branched ratio.

Alcohols isolated from the neutral fraction were examined by GC and GC-MS. In GC reproduced in Fig. 4 are given the result of analyses; molecular weight determination of major peaks (C_{20} to C_{26}) by GC-MS and comparison of retention times with authentic normal alcohols. As was the case in fatty acids the alcohols constitute predominantly of branched ones, probably of terminal iso-propyl and/or *sec*-butyl structure since MS were similar to those of normal alcohols and showed relatively high peak at *m/e* 70.[13] The presence of very small amount of unsaturated counterpart was indicated by GC of the sample separated by silver nitrate complexing TLC.[14]

Fraction III

This fraction contained almost pure muscone (3-methylcyclopentadecanone) by comparison of IR, GC and TLC with those of authentic sample.[15]

Fraction IV

MS showed that this fraction contained fatty alcohols, the composition of which was almost the same as that obtained from Fraction II.

Fraction V

By GC and GC-MS this fraction was found to contain cholesterol and small amount of cholest-4-en-3-one (about 10% of the former).

Fraction VI

Crystalline cholesterol was isolated and identified by MS, TLC, and GC.

Fraction VII

The gas chromatogram of trimethylsilation product is shown in Fig. 5. Assignment of peaks was made based on the following analyses of Fraction VII-1 to VII-6.

Fraction VII-1

Two major components appeared in GC (Fig. 6) were proved to be 5α-androstane-3,17-dione and 5β-androstane-3,17-dione by comparison of retention times with authentic samples and GC-MS.

Fraction VII-2

The gas chromatogram showed a partially resolved peak as shown in Fig. 7. It was found to be a mixture of androst-4-ene-3,17-dione and androsta-4,6-diene-3,17-dione by GC-MS and UV maxima at 240 and 282 nm of the mixture.

Fraction VII-3

The gas chromatogram showed one peak, which on trimethylsilation was resolved into

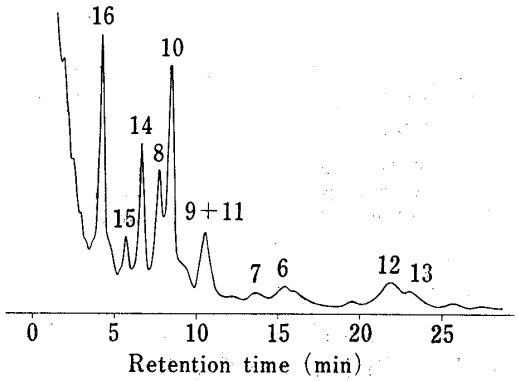

Fig. 5. Gas Chromatogram of Trimethylsilated Fraction VII

column: OV-17, 210° The numbering of peaks corresponds to the one in Table II.

13) R.A. Brown, W.S. Young, and N.N. Nicolaides, *Anal. Chem.*, **26**, 1653 (1954).
14) E.J. Singh and L.L. Gershbein, *J. Chromatog.*, **29**, 229 (1967).
15) E. Yoshii and S. Kimoto, *Chem. Pharm. Bull.* (Tokyo), **17**, 629 (1969).

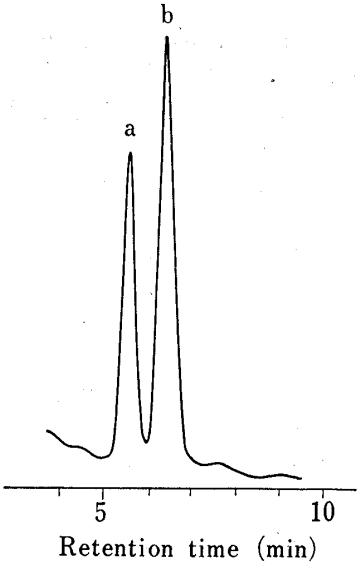

Fig. 6. Gas Chromatogram of Fraction VII-1

column OV-17, 235°
a: 5β-androstane-3,17-dione
b: 5α-androstane-,317-dione

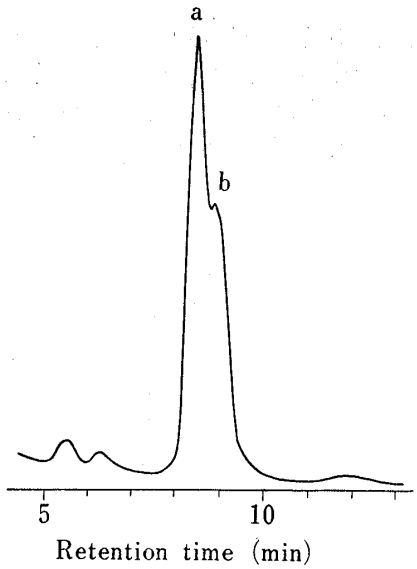

Fig. 7. Gas Chromatogram of Fraction VII-2

column: OV-17, 235°
a: androst -4-ene-3,17-dione
b: androsta-4,6-diene-3,17-dione

two peaks as shown in Fig. 8. Interpretation of MS data obtained by GC-MS of both free and TMS ethers, retention times, and TLC behaviors (solvent system II and III) indicated that this fraction contains 3α-hydroxy-5α-androstan-17-one and androst-5-en-3β-ol-17-one.

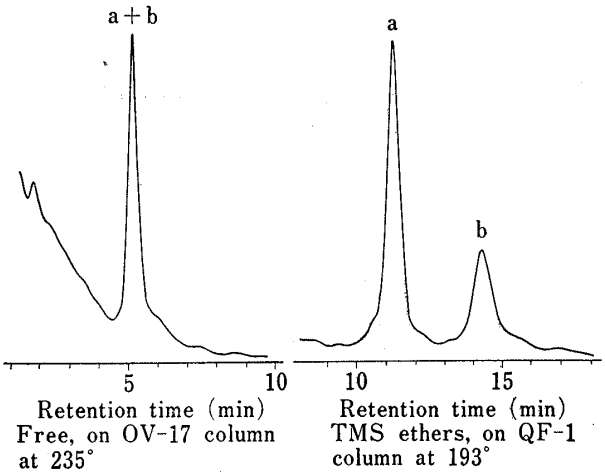

Fig. 8. Gas Chromatogram of Fraction VII-3

a: 3α-hydroxy-5α-androstan-17-one
b: 3α-hydroxy-androst-5-en-17-one

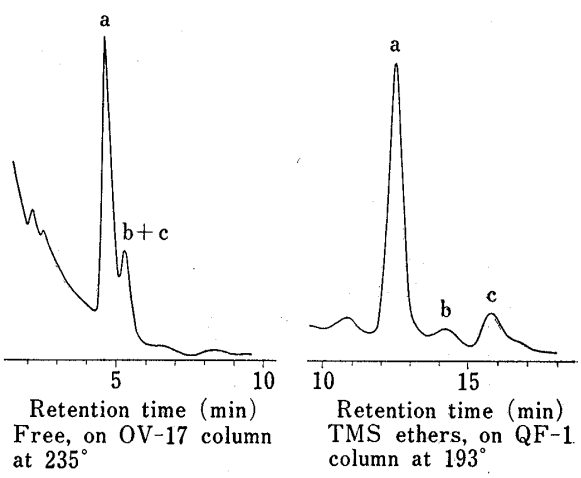

Fig. 9. Gas Chromatogram of Fraction VII-4

a: 3α-hydroxy-5β-androstan-17-one
b: 3β-hydroxy-androst-5-en-17-one
c: 3β-hydroxy-5α-androstan-17-one

Fraction VII-4

The gas chromatograms of this fraction are shown in Fig. 9. The GC-MS data and comparison of retention times with authentic samples showed that this fraction constitutes of 3α-hydroxy-5β-androstan-17-one[16] as major component accompanied by small amount of 3β-hydroxy-5α-androstan-17-one and 3β-hydroxy-androst-5-en-17-one.

16) E. Elisberg, H. Vanderhaeghe, and T.F. Gallagher, *J. Am. Chem. Soc.*, **74**, 2814 (1952).

Fraction VII-5

It was indicated by GC-MS that this fraction contains an androstanediol. It was oxidizable with chromic acid to give 5α-androstane-3,17-dione. Since by partial oxidation the formation of 3β-hydroxy-5α-androstane-17-one was realized on GC and TLC, this diol should be either 3β,17β-dihydroxy- or 3β,17α-dihydroxy-5α-androstane. The choice of the latter

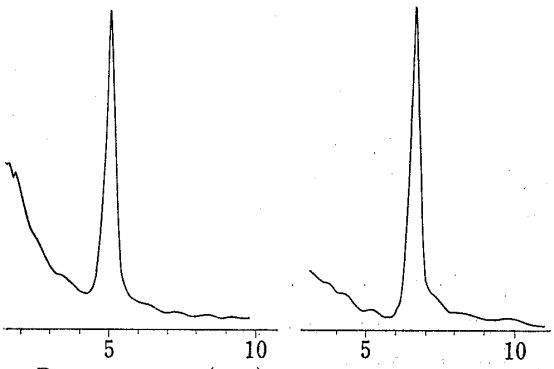

Fig. 10. Gas Chromatogram of Fraction VII-5 (5α-androstane-3β,17α-diol)

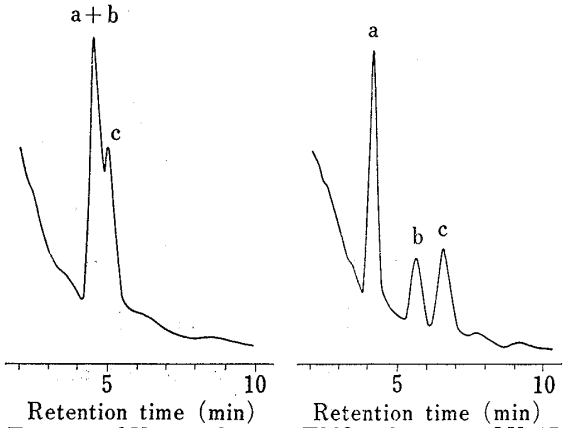

Fig. 11. Gas Chromatogram of Fraction VII-6

a: 5β-androstane-3α,17α-diol
b: 5β-androstane-3α,17β-diol
c: 5α-androstane-3β,17α-diol

Table II. Chromatographic Data of identified Substances in Musk

		Rf (TLC) Solvent system[a]			Retention time (min)[a]		
					Free	TMS ether	
		II	I (IV)	III	OV-17[b]	OV-17[c]	QF-1[d]
1.	Cholesterol esters[e]	0.85	0.65	0.77			
2.	Wax[f]	0.87	0.51	0.74			
3.	Muscone	0.79	0.35	0.69			
4.	Cholesterol	0.49	0.05	0.48	17.6	(14.2)	
5.	Cholest-4-en-3-one	0.62		0.65	24.7		
6.	5α-Androstane-3,17-dione	0.50		0.60	6.5(16.0)		
7.	5β-Androstane-3,17-dione	0.46		0.59	5.6(13.6)		
8.	3α-Hydroxy-5α-androstan-17-one	0.36		0.40	5.1	7.6	11.3
9.	3β-Hydroxy-androst-5-en-17-one	0.34		0.37	5.3	10.5	14.2
10.	3α-Hydroxy-5β-androstan-17-one	0.27		0.35	4.7	8.4	12.5
11.	3β-Hydroxy-5α-androstan-17-one	0.32		0.36	5.3	10.7	15.8
12.	Androst-4-ene-3,17-dione	0.36		0.51	8.5		
13.	Androsta-4,6-diene-3,17-dione	0.36		0.53	8.9		
14.	5α-Androstane-3β,17α-diol	0.26	(0.27)	0.29	5.0	6.7	5.6
15.	5β-Androstane-3α-17β-diol	0.19	(0.19)	0.26	4.6	5.7	5.2
16.	5β-Androstane-3α,17α-diol	0.11	(0.14)	0.21	4.4	4.2	3.8
	[5α-Cholestane				6.3(15.9)		10.9]

a) See experimental section. *b*) At 235° except the values in parentheses at 210°. *c*) At 210° except the value in parenthesis at 235°. *d*) At 193° *e*) Fraction I *f*) Fraction II

isomer was made by elimination of the former, authentic sample of which showed different retention time (free 5.2 min, TMS ether 7.6 min). This conclusion was also supported by the observed steroid number (SN) of TMS ether, 24.45 (OV-17, reported 24.45).[17]

Fraction VII-6

The GC-MS indicated that this fraction also contains androstandiols. GC of trimethylsilated product showed three peaks (Fig. 11); the last one (t_R=6.7 min) of which was assigned to 5α-androstane-3β,17α-diol appeared in the previous fraction. The remaining two peaks were ascribed to those due to 3α,17α- and 3α,17β-diols of 5β-androstane, since on chromic acid oxidation they were transformed into 5β-androstane-3,17-dione while partial oxidation afforded 3α-hydroxy-5β-androstan-17-one as major product. Retention times of TMS ethers were determined to be 4.2 min for 3α,17α-diol and 5.7 min for 3α,17β-diol by referring that of authentic latter isomer.[16] Graphically obtained SN (OV-17), 23.15 for the former and 24.05 for the latter, were also consistent with this assignment (reported,[17] 23.20 and 24.05 respectively).

The components of musk identified in the present investigation and their chromatographic data employed for the foregoing discussions are given in Table II. Among them, androstane derivatives responsible for the male hormonal activity[4] could easily be identified by running GC (see Fig. 5) of more polar fraction than cholesterol obtained silica gel TLC using benzene as developing solvent. Application of this method for the evaluation of quality of the musk is without doubt far more reliable than what have been adopted in Japanese Pharmacopea, and it will be a subject of further investigation. Moreover, the presence of long chain carboxylic acid esters of cholesterol and higher alcohols should be emphasized, and analyses of these characteristic components will also contribute to quality estimation.

Acknowledgement The authors are grateful to Professors M. Kimura and I. Waki of this university who provided ether extract of the musk, and also to Kokando Co., Ltd. for performing programmed gas chromatography.

17) F. Berthou, L. Bardou, and H.H. Floch, *J. Chromatog.*, 93, 149 (1974).