GAS CHROMATOGRAPHY - MASS SPECTROMETRY OF CATECHOL ESTROGENS

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ABSTRACT

The gas chromatographic and mass spectrometric properties of 19 catechol estrogens and catechol estrogen methyl ethers are reported. The gas chromatographic behaviour of the TMS-derivatives on the stationary phases OV-1, OV-3, OV-7, and OV-17 is examined and correlated with their molecular weight, shape, and polarity. The characteristic mass spectrometric features of the compounds result from the aromatic ring A, which is able to stabilize positive charge within the molecular ions. Consequently the molecular ions form the base peaks of the spectra. Fragmentation patterns highly specific for the catechols as well as for their monomethyl and dimethyl ethers are discussed and substantiated by determination of metastable ions and high resolution mass measurements.

INTRODUCTION

The formation of catechol estrogens and their methyl ethers is a major route of estrogen metabolism in man [1-7] as well as in laboratory animals [8-12]. Although these ring A-hydroxylation and methyla-

tion reactions cause loss of estrogenic activity of the steroid hormones [13] the metabolites formed have been shown to interact with other hormones, for example KNUPPEN *et al.* [14-16] demonstrated that catechol estrogens act as strong inhibitors of enzymic catecholamine deactivation. Furthermore GELBKE *et al.* [17] suggested recently that determination of 2-hydroxylated estrogens might be a useful parameter for monitoring abnormal pregnancies.

Relatively little information is available on the gas chromatographic and mass spectrometric behaviour of this important group of steroid hormone metabolites. Combined gas liquid chromatography-mass spectrometry (GLC-MS) is invaluable in the separation and identification of complex mixtures of estrogen metabolites; moreover the outstanding technique of fragmentography [18] renders possible highly specific quantitative determinations of steroids within the picogram region [19-22]. A necessary prerequisite for these qualitative and quantitative analyses is a knowledge of the GLC-retention values and of the MS-fragmentation patterns which are examined in the present study for 19 ring A-substituted estrogens.

MATERIALS AND METHODS

Steroids*: estrone, estradiol, and estriol were gifts from Schering AG, Berlin. 2-Hydroxyestrone, 4-hydroxyestrone, 2-hydroxyestradiol, 4-hydroxyestradiol, 2-hydroxyestriol, and 4-hydroxyestriol were prepared according to GELBKE *et al.* [23]. 2-Methoxy-

*See nomenclature list

estrone, 2-hydroxyestrone 3-methyl ether, 2-methoxyestradiol, 2-hydroxyestradiol 3-methyl ether, 2-methoxyestriol, and 2-hydroxyestriol 3-methyl ether were synthesized using the method of FISHMAN *et al.* [24]. 2-Hydroxyestrone 2,3-dimethyl ether, 4-hydroxyestrone 3,4-dimethyl ether, 2-hydroxyestradiol 2,3-dimethyl ether, and 2-hydroxyestriol 2,3-dimethyl ether were obtained by treatment of the corresponding catechol compounds with diazomethane as described [25]. 4-Hydroxyestrone 3-methyl ether, 4-hydroxyestrone 4-methyl ether, and 4-hydroxyestradiol 3-methyl ether [26] were kindly supplied by Prof. Dr.R.Knuppen, Lübeck.

Prior to analysis hydroxy-steroids were converted to their TMS-derivatives by reaction with N,O-bis(trimethylsilyl)-trifluoroacetamide containing 1% chlorotrimethylsilane (W. Günther Analysentechnik, Düsseldorf) as described previously [19].

Gas liquid chromatography was carried out using a Pye 104 gas chromatograph (Philips, Hamburg) equipped with a flame ionisation detector. Glass columns (2.1 m × 3 mm ID) were packed with either 3% OV-1 on 100/120 chromosorb W HP, 3% OV-3 on 100/120 chromosorb W HP, 3% OV-7 on 100/120 chromosorb W HP, or 3% OV-17 on 100/120 chromosorb W HP. The precoated supports were purchased from W. Günther Analysentechnik, Düsseldorf.

GLC-conditions: column temperature 215^0 C; detector temperature 250^0 C; carrier gas flow 25 ml nitrogen per min.

Low resolution mass spectra were recorded on a LKB 9000 gas chromatograph-mass spectrometer (LKB Produkter, Stockholm). Operation conditions: column temperature 215^0 C; carrier gas flow 25 ml per min; temperature of molecular separator 230^0 C; ion source temperature 250^0 C; ionisation energy 70 eV. High resolution mass spectra were recorded

Table 1. GLC of TMS-derivatives of estrogens on 3% OV-1, OV-3, OV-7, and OV-17 at 215° C. GLC-retention values are given relative to estriol-TMS.

Steroid	Relative retention values on the phases OV-1	OV-3	OV-7	OV-17
estriol	1	1	1	1
2-hydroxyestrone	0.66	0.77	0.80	1.08
2-hydroxyestrone 3-methyl ether	0.61	0.73	0.85	1.23
2-hydroxyestrone 2-methyl ether	0.63	0.75	0.86	1.25
2-hydroxyestrone 2,3-dimethyl ether*	0.59	0.72	0.86	1.44
4-hydroxyestrone	0.72	0.86	0.95	1.23
4-hydroxyestrone 4-methyl ether	0.53	0.68	0.75	1.02
4-hydroxyestrone 3-methyl ether	0.60	0.77	0.88	1.50
4-hydroxyestrone 3,4-dimethyl ether*	0.45	0.63	0.74	1.19
2-hydroxyestradiol	0.82	0.82	0.80	0.79
2-hydroxyestradiol 3-methyl ether	0.72	0.78	0.81	0.87
2-hydroxyestradiol 2-methyl ether	0.76	0.81	0.84	0.90
2-hydroxyestradiol 2,3-dimethyl ether	0.69	0.78	0.84	1.05
4-hydroxyestradiol	0.90	0.92	0.92	0.92
4-hydroxyestradiol 3-methyl ether	0.76	0.81	0.84	0.92
2-hydroxyestriol	1.58	1.48	1.48	1.33
2-hydroxyestriol 3-methyl ether	1.43	1.41	1.48	1.55
2-hydroxyestriol 2-methyl ether	1.47	1.48	1.54	1.62
2-hydroxyestriol 2,3-dimethyl ether	1.30	1.41	1.55	1.86
4-hydroxyestriol	1.82	-	-	1.57

*These steroids are measured as free compounds

on a AEI MS30 gas chromatograph-mass spectrometer coupled to a DS30 data system (AEI Scientific Apparatus Ltd., Manchester) under the operation conditions described above.

RESULTS AND DISCUSSION

In the course of the present investigation trimethyl silyl ethers of catechol estrogens proved to be the most suitable derivatives for gas chromatography as well as for mass spectrometry. In addition to being easily prepared, the silylated derivatives exhibit low polarity and high thermal stability which results in symmetrical GLC-peaks and high intensities of molecular ions. The gas chromatographic retention data of the estrogens analysed on four stationary phases (OV-1, OV-3, OV-7, OV-17) are compiled in Table 1.

The relative retention times of the TMS-steroids depend upon molecular weight as well as shape and polarity of the molecules. Molecular weight predominantly influences the gas chromatographic behaviour on OV-1 which is a non polar stationary phase consisting of a methylsilicone polymer. The higher the molecular weight of the steroid derivatives the longer the retention time on OV-1. In addition, the effect of molecular shape is shown by the retention times of 4-hydroxyestrone, 4-hydroxyestradiol, and 4-hydroxyestriol (Fig. 1). The 4-trimethylsiloxy groups are stretched out resulting in longer retention times than the corresponding 2-substituted compounds.

The stationary phases OV-3, OV-7, and OV-17 differ from OV-1 by increasing numbers of phenyl groups within the silicone polymer resulting in an increase of polarity. Thus OV-17 separates steroids according to their content of polar groups, while the properties of

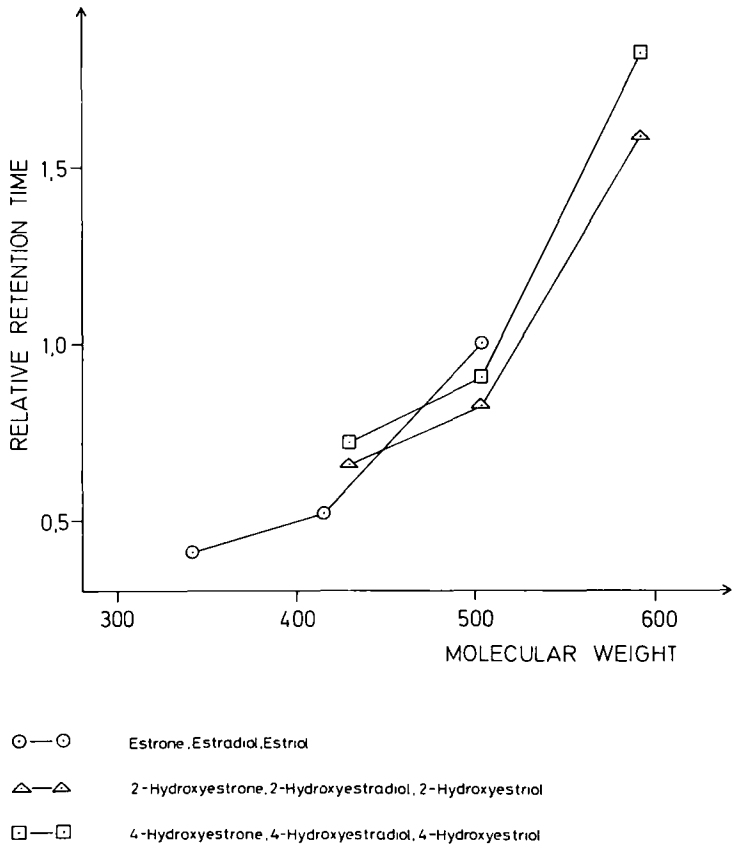


Fig. 1. Effect of molecular weight and molecular shape of TMS-steroids on retention time relative to estriol-TMS

OV-3 and OV-7 may be situated between those of OV-1 and OV-17. For example the retention values of 2-hydroxyestrone increase due to the 17-oxo group from 0.66 on OV-1 up to 1.08 on OV-17 whereas the corresponding data for 2-hydroxyestradiol remain virtually constant on all four phases.

The chromatographic properties of methylated catechol estrogens

are of special interest. Compared with the trimethylsilyl ethers the methoxy groups behave like weakly polar groups. This is demonstrated in Table 1. Within the series 2-hydroxyestrone, 2-hydroxyestrone 2-methyl ether, 2-hydroxyestrone 3-methyl ether, and 2-hydroxyestrone 2,3-dimethyl ether the relative retention times on OV-1 decrease from 0.66 to 0.59 with decreasing molecular weights, whereas they increase on OV-17 columns from 1.08 to 1.44 due to the polar effect of the methyl ethers. The same phenomenon can be observed with 2-hydroxyestradiol and 2-hydroxyestriol by comparing the catechols with their monomethyl and dimethyl ethers.

With the 3,4-substituted steroids the situation is somewhat complicated. Although retention values follow molecular weight on OV-1, on OV-17 the steric effect of the bulky 4-trimethylsiloxy group even overcomes the influence of the weak polar methyl ethers. Thus 4-hydroxyestrone and 4-hydroxyestrone 3-methyl ether show longer retention times on OV-17 than do the corresponding 4-methylated compounds, 4-hydroxyestrone 4-methyl ether and 4-hydroxyestrone 3,4-dimethyl ether.

Table 2 lists the mass spectrometric data of the catechol estrogens investigated. The characteristic mass spectral feature of these compounds is the molecular ion, usually the base peak in the spectrum. This is due to the resistance of aromatic ring A to fragmentation induced by electron impact and to the extraordinary power of catechol trimethylsilyl and methyl ethers to stabilize positive charge. Small M-15 peaks exist in all spectra; although loss of the C-18 methyl radical is possible, the most probable site of methyl cleavage is at the silicon atom [27]. The mass

Table 2. Mass spectrometric data of TMS-derivatives of catechol estrogens. m/e Values are given in normal figures and the corresponding relative intensities in *Italics*.

Steroid	M^+	M-15	M-28	M-30	M-31	M-56	M-57	M-85	M-89	M-124	Other fragments
2-hydroxyestrone	430	415	402			374	373	345	341	306	73
	100	*5*	*1*			*1*	*1*	*3*	*5*	*6*	*75*
2-hydroxyestrone 3-methyl ether	372	357		342	341			287		248	73
	100	*6*		*35*	*2*			*5*		*5*	*35*
2-hydroxyestrone 2-methyl ether	372	357		342	341			287		248	73
	100	*7*		*33*	*2*			*6*		*3*	*30*
2-hydroxyestrone 2,3-dimethyl ether*	314	299	286	284	283	258	257	229	225	190	97
	100	*5*	*2*	*1*	*4*	*2*	*3*	*9*	*1*	*12*	*10*
4-hydroxyestrone	430	415	402			374	373	345	341	306	73
	100	*5*	*3*			*2*	*2*	*7*	*13*	*6*	*82*
4-hydroxyestrone 4-methyl ether	372	357	344	342	341	316	315	287	283	248	73
	100	*9*	*3*	*33*	*9*	*1*	*2*	*9*	*1*	*8*	*27*
4-hydroxyestrone 3-methyl ether	372	357	344	342	341	316	315	287	283	248	73
	100	*22*	*6*	*61*	*2*	*1*	*1*	*5*	*1*	*4*	*25*
4-hydroxyestrone 3,4-dimethyl ether*	314	299	286	284	283	258	257	229	225	190	97
	100	*2*	*1*	*2*	*9*	*3*	*4*	*13*	*1*	*12*	*13*

*These steroids are measured as free compounds

Table 2. (cont.)

Steroid	M^+	M-15	M-30	M-90	M-131	M-185	$\frac{m}{e}$129	$\frac{m}{e}$73	Other fragments	
2-hydroxyestradiol	504	489		414	373	319	129	73	205	267
	100	*6*		*9*	*8*	*20*	*71*	*79*	*24*	*27*
2-hydroxyestradiol 3-methyl ether	446	431	416	356	315	261	129	73	209	218
	100	*6*	*7*	*3*	*14*	*8*	*12*	*60*	*8*	*9*
2-hydroxyestradiol 2-methyl ether	446	431	416	356	315	261	129	73	209	218
	100	*6*	*7*	*3*	*14*	*8*	*10*	*60*	*10*	*8*
2-hydroxyestradiol 2,3-dimethyl ether	388	373		298	257	208	129	73	151	203
	100	*3*		*5*	*30*	*14*	*28*	*60*	*20*	*14*
4-hydroxyestradiol	504	489		414	373	319	129	73	415	285
	100	*5*		*2*	*9*	*3*	*8*	*36*	*2*	*9*
4-hydroxyestradiol 3-methyl ether	446	431	416	356	315	261	129	73	218	261
	100	*14*	*13*	*5*	*25*	*8*	*13*	*43*	*7*	*8*

Table 2. (cont.)

Steroid	M^+	M-15	M-90	M-118	M-144	M-159	M-180	M-193	M-207	$\frac{m}{e}$147	$\frac{m}{e}$129	$\frac{m}{e}$73
2-hydroxyestriol	592	577	502	474	448	433	412	399	385	147	129	73
	68	*3*	*2*	*2*	*4*	*4*	*2*	*3*	*4*	*25*	*5*	*100*
2-hydroxyestriol	534	519	444	416	390	375	354	341	327	147	129	73
3-methyl ether	*100*	*4*	*4*	*7*	*10*	*14*	*6*	*11*	*11*	*14*	*19*	*98*
2-hydroxyestriol	534	519	444	416	390	375	354	341	327	147	129	73
2-methyl ether	*100*	*3*	*1*	*6*	*8*	*12*	*4*	*7*	*10*	*10*	*20*	*90*
2-hydroxyestriol	476	461	386	358	332	317	296	283	269	147	129	73
2,3-dimethyl ether	*100*	*1*	*5*	*11*	*7*	*21*	*6*	*19*	*15*	*15*	*18*	*75*
4-hydroxyestriol	592	577	502	474	448	433	412	399	385	147	129	73
	46	*2*	*3*	*4*	*4*	*6*	*3*	*6*	*5*	*14*	*12*	*100*

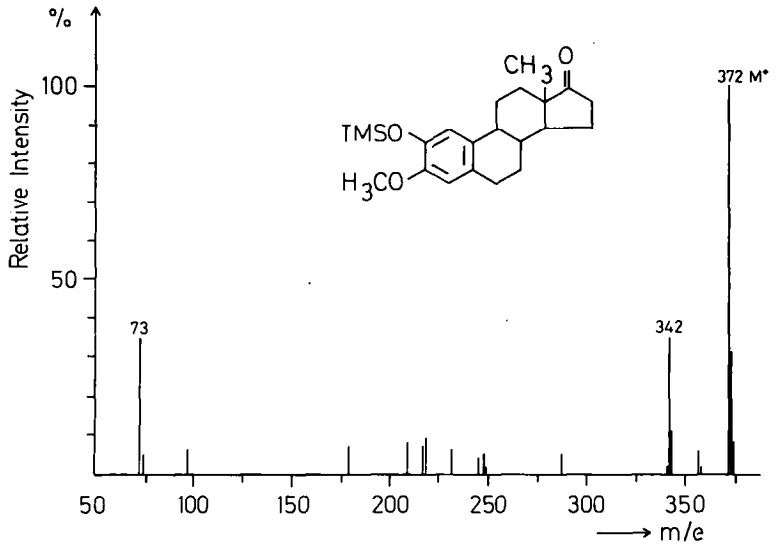


Fig. 2. Mass spectrum of 2-hydroxyestrone 3-methyl ether

spectra of catechol estrogen monomethyl ethers are characterized by ions at M-30 which are the most important peaks in the fragmentation patterns of the isomeric monomethyl ethers of 2-hydroxyestrone and 4-hydroxyestrone (Fig. 2). High resolution measurements established that the M-30 can certainly be attributed to the loss of 2 methyl groups. As summarized in table 3 the loss of CH_2O can be excluded.

Table 3 High resolution mass measurement of the M-30 peaks of the isomeric monomethyl ethers of 2-hydroxyestrone and 4-hydroxyestrone (as TMS-derivatives)

Steroid	found for M-30	calculated for $C_{20}H_{26}O_3Si$	calculated for $C_{21}H_{30}O_2Si$
2-hydroxyestrone 2-methyl ether	342.1666	342.1651	342.2015
2-hydroxyestrone 3-methyl ether	342.1638	342.1651	342.2015
4-hydroxyestrone 3-methyl ether	342.1635	342.1651	342.2015
4-hydroxyestrone 4-methyl ether	342.1642	342.1651	342.2015

Table 4. Metastable ions in the mass spectra of mono- and dimethyl ethers of 2-hydroxyestrone and 4-hydroxyestrone (TMS derivatives) Theoretical values are calculated using the formula $m^* = \frac{m_2^2}{m_1}$

Transition m_1	m_2	m/e calcd.	m/e found
372	342	314,42	314,5
357	342	327,63	327,8
314	283	255,06	255,2

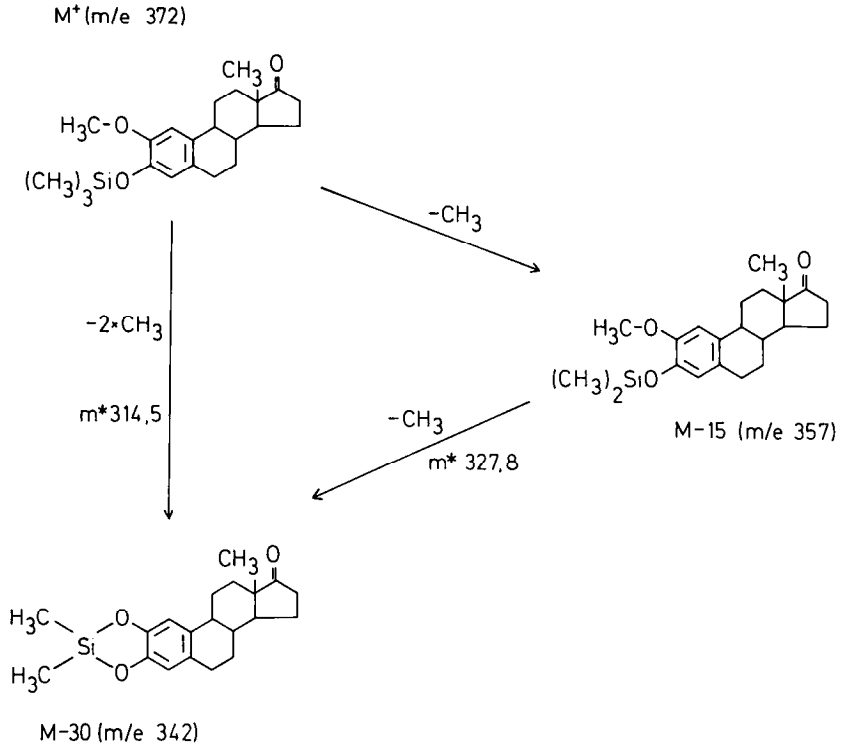
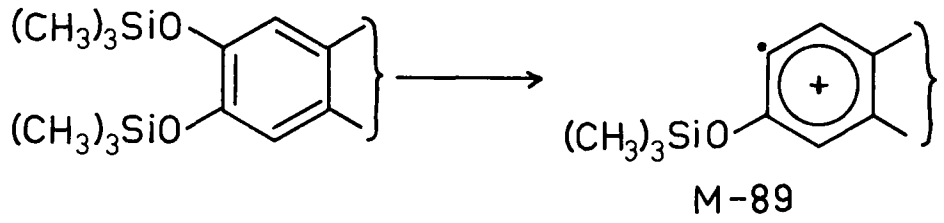


Fig. 3. M-15 and M-30 fragments of the TMS-ether of 2-hydroxyestrone 2-methyl ether; formation of metastable ions at m^* 314,5 and m^* 327,8

The expulsion of two methyl groups leads to a cyclic ion, the proposed structure of which is shown in Figure 3. The occurrance of metastable ions (Table 4) indicates that m/e 342 is formed by two different ways: 1. Simultaneous loss of two methyl groups, one from the methoxy- and the second from the TMS-moiety, yielding a metastable ion m^* at m/e = 314,5 (calculated m^* = 314,41). 2. Subsequent loss of two methyl groups via the M-15, the second step (357 → 342) of this process producing a metastable m^* at m/e = 327,8 (calculated m^* = 327,63). The data given above leave no doubt, that M-30 is the result of a rearrangement of the methoxy and trimethylsiloxy groups at ring A of the estrogen molecule. The suggestion that the consecutive expulsion of H_2 and C_2H_4 could be the source of this fragment [28] is not in accordance with our findings. The spectra of the isomeric monomethyl ethers of 2-hydroxyestradiol, 4-hydroxyestradiol, and 2-hydroxyestriol exhibit peaks of lower intensities at M-30, since ring D-induced fragmentation acquires increasing significance.

Within the series of catechol estrogen monomethyl ethers only 4-methoxyestrone shows a M-31, which is a major peak in the spectra of 2-hydroxyestrone 2,3-dimethyl ether and 4-hydroxyestrone 3,4-dimethyl ether (Fig. 4). This type of fragment arises by expulsion of a methoxy radical as confirmed by the metastable ions (cf. Table 4).

Characteristic for catechol estrogen TMS-ethers are peaks at M-89 which are attributed to the loss of trimethylsiloxy-radicals.



M-89

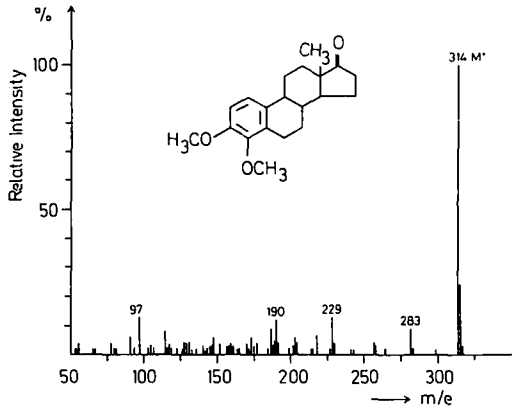


Fig. 4. Mass spectrum of 4-hydroxyestrone 3,4-dimethyl ether

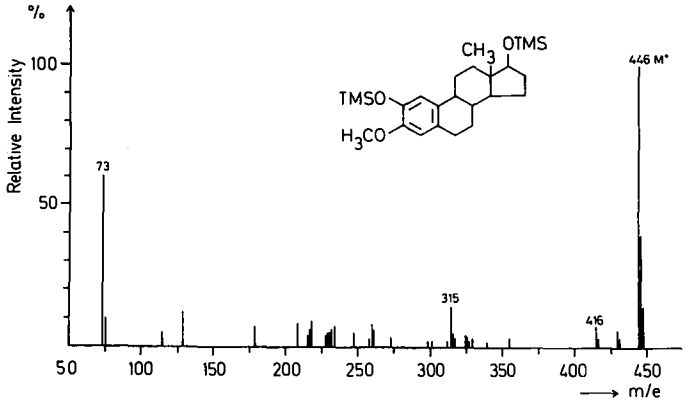


Fig. 5. Mass spectrum of 2-hydroxyestradiol 3-methyl ether

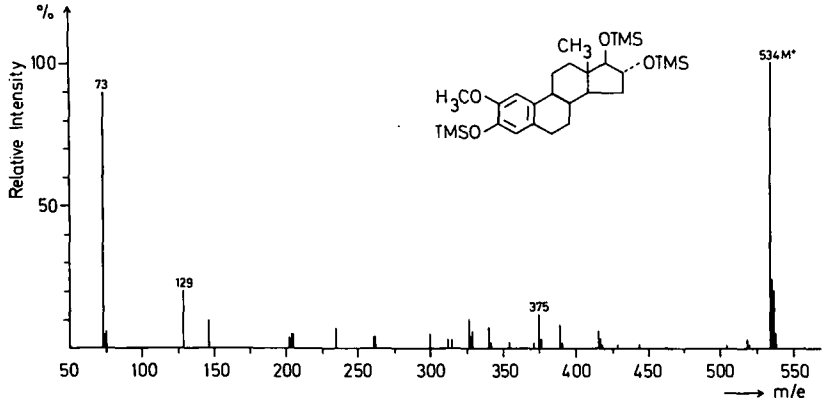


Fig. 6. Mass spectrum of 2-hydroxyestriol 2-methyl ether

The remainder of the fragmentation pattern of the steroids investigated is governed by the type of functional groups at ring D of the molecules. Thus, the spectra of all 17-oxo-estrogens show peaks at M-56, M-57, M-85, and M-124 which are generated following reaction mechanisms analogous to those described for estrone methyl ether [29]. 17β-Hydroxysteroid TMS ethers expulse ring D fragments yielding peaks at m/e 129 and M-131 (Fig.5). Similar results are observed for 5α-androstane-17β-ol TMS ether [30]. Ring A-substituted estriols (Fig.6) are degraded in the same way as estriol, the mass spectrum of which is discussed in detail by ADLERCREUTZ and LUUKKAINEN [31].

It can be concluded that the trimethylsilyl derivatives of catechol estrogens and their methyl ethers show highly characteristic fragmentation patterns in mass spectrometry. Combined gas chromatography-mass spectrometry can be recommended as a powerful analytical tool in biochemical and endocrinological investigations involving catechol estrogens, as demonstrated previously [25]. Furthermore the outstanding gas chromatographic and mass spectrometric features of the compounds studied in this paper make them attractive for quantitative determinations using the high specific and sensitive method of fragmentography [19-21].

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Nomenclature list

2-Hydroxyestrone	2,3-dihydroxy-1,3,5(10)-estratrien-17-one
4-Hydroxyestrone	3,4-dihydroxy-1,3,5(10)-estratrien-17-one
2-Hydroxyestradiol	1,3,5(10)-estratriene-2,3,17β-triol
4-Hydroxyestradiol	1,3,5(10)-estratriene-3,4,17β-triol
2-Hydroxyestriol	1,3,5(10)-estratriene-2,3,16α,17β-tetrol
4-Hydroxyestriol	1,3,5(10)-estratriene-3,4,16α,17β-tetrol
2-Methoxyestrone	2-hydroxyestrone 2-methyl ether
2-Methoxyestradiol	2-hydroxyestradiol 2-methyl ether
2-Methoxyestriol	2-hydroxyestriol 2-methyl ether
TMS-derivative	trimethylsilyl derivative