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CID Mass Spectrometric Characterization of Some Epimeric Anabolic Androgenic Steroids

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Abstract

A GC/tandem MS with collision-induced dissociation (CID) technique is described for the characterization of the 5α- and 5β-isomers of some anabolic androgenic steroids (AAS). The most characteristic type of fragmentation of the 5-isomers tested is the loss of water and the loss of a methyl group from the molecular ions. The 5α-isomers, such as androsterone, 5α-androstane-3α,17β-diol and 5α-androstane-3α,11β-diol-17-one prefer to lose a methyl radical from their molecular ions first, but the corresponding 5β-isomers show that the loss of water from their molecular ions is the dominating step in their fragmentation. The increase of the collision energy results in more difference between the mass spectra of them:. 5β-isomers gave much more intensive fragments than 5α-isomers. The mass spectra are discussed. The trimethylsilyl (TMS) derivatives of these substances show some different behavior. Three pairs of 5-epimeric AAS and one pair of per TMS derivatives can be characterized by their CID mass spectra. At last some small peaks between two huge interfering peaks in a routine urine sample could be identified with CID mass spectra.

Introduction

In most cases of doping control analysis the retention time is the major tool to distinguish the epimeric anabolic androgenic steroids (AAS). An OV-1 column with 0.11 μm of film thickness and a slow raised temperature program with about 3 °C/min are used to separate such pairs of epimeric AAS(1). The normal EI mass spectra do not show significant differences between these epimeric AAS. In some cases, when the retention time is little shifted, e.g. due to a huge amount or interference from biomatrix etc., we need indeed to identify them by mass spectra.

The potential use of mass spectrometry for solving structural problems in a number of steroids was demonstrated by some early publications, in which mass spectrometry worked at low electron energy (-9 to -15 eV) or with other ionization methods (chemical ionization etc.) to determine the molecular weight (2). Most of the later studies on such epimeric structures of steroids were performed with various different mass spectrometric techniques for structure study (3).

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There are several reasons for developing a mass spectrometric method to characterize certain epimeric steroids. Such epimeric steroids as androsterone and etiocholanolone, 5α-androstane-3α,17β-diol and 5β-androstane-3α,17β-diol, 11β-OH-androsterone and 11β-OH-etiocholanolone are often involved in our routine analysis and show nearly identical EI mass spectra. These epimeric steroids are also important markers for detection of doping with endogenous steroids. A rapid, reliable mass spectrometric method capable of characterizing the epimeric steroids in urine will be not only helpful for forensic confirmation in our routine work but also for an excretion study with a new steroid agent because some of the AAS with an 4-en-3-one structure metabolite to different 5α-/5β-reduced isomers. GC/tandem MS with collision-induced dissociation (CID) technique was used to study the difference of daughter ion mass spectra of these epimeric steroids with and without MSTFA derivatization.

Experimental

A gas chromatograph model 5890 series II plus (Hewlett-Packard) was connected to a TSQ-7000 (Finnigan). The separation was carried out with a HP 1 column (17m, 0.2 mm I.D., 0.11 μm film thickness). The injector operated in split mode (1:10 split ratio) and the temperature was maintained at 280°C. The temperature program was: 0 min 180°C, + 3.3°C/min, 0 min 231°C, + 30°C/min, 2 min 310°C. Helium was used as carrier gas with a flow rate of 0.8 ml/min (at 180°C). The pressure was kept constant automatically during the runs. The transfer line was set to 280°C. For GC/MS/MS the first MS worked in EI mode (electron energy 70 eV). The second MS worked in daughter mode with a scan range from m/z 50 to 500. The manifold was set to 70°C, the ion source to 180 °C, the conversion dynode to 15 kV, the scan rate to 500 amu/s. The emission current was 400 μA, the electron multiplier voltage in the range of 1400 - 1500. PFTBA was used as calibrator for tuning. Collision gas was argon at 1.8 - 2 mTorr. The collision energy was changed in the range of 5 - 30 V. The correction factor (MSMSC) was 0.7 - 1.0.

The extraction and derivatization procedures used were the same as our routine procedure IV for the total fraction. All steroids were obtained from Steraloids Inc (USA), MSTFA was purchased from Sigma (USA).

Results and Discussion

Under our experimental conditions the epimeric steroids studied can be separated by their retention times. Without derivatization the 5β-isomers are eluted in GC earlier than the 5α-isomers, but after derivatisation with MSTFA, the order of elution is converted.

Fig.1 shows the CID spectra of 5α- and 5β-androstan-3α-ol-17-one with a collision energy of -10 eV. The molecular ion m/z 290 was selected as parent ion. 5α-Androstan-3α-ol-17-one produces the molecular ion (m/z 290, base peak) and the fragments m/z 275 raised

Tab.1 Retention Times of the Epimeric Steroids without and with MSTFA Derivatisation

Substances	Retention Time (min)	Relative RT
5α-androstan-3α-ol-17-one	9.24	0.61
5β-androstan-3α-ol-17-one	8.79	0.58
5α-androstane-3α,17β-diol	9.49	0.63
5β-androstane-3α,17β-diol	8.93	0.59
5α-androstane-3α,11β-diol-17-one	12.65	0.83
5β-androstane-3α,11β-diol-17-one	11.93	0.79
5α-androstan-3α-ol-17-one, bis-*O*-TMS	10.93	0.72
5β-androstan-3α-ol-17-one, bis-*O*-TMS	11.14	0.73
5α-androstane-3α,17β-diol, bis-*O*-TMS	11.26	0.74
5β-androstane-3α,17β-diol, bis-*O*-TMS	11.36	0.75
5α-androstane-3α,11β-diol-17-one, tris-*O*-TMS	13.75	0.91
5β-androstane-3α,11β-diol-17-one, tris-*O*-TMS	13.92	0.92

ISTD: Methyltestosterone, bis-*O*-TMS, retention time=15.16

from the loss of a methyl radical from the molecular ion, with smaller intensity m/z 272 raised from the loss of water from the molecular ion and m/z 246, while the CID spectrum of 5β-androstan-3α-ol-17-one shows the base peak m/z 272 (m/z 244 is considered as artifact), the molecular ion m/z 290, m/z 246 and m/z 257 raised from the loss of a methyl group from m/z 272. That the ion m/z 275 can not be found in the CID spectrum of 5β-androstan-3α-ol-17-one may mean that the loss of a methyl radical from the molecular ion can not happen easily.

The similar behavior is observed in the CID spectra of 5α- and 5β-androstane-3α,17β-diol with a collision energy of -10 eV (Fig. 2). The molecular ion m/z 292 was selected as parent ion. The base peak m/z 277 in the CID spectrum of 5α-androstane-3α,17β-diol results from the loss of a methyl radical from the molecular ion. By contraries, m/z 274 is the base peak in the CID mass spectrum of 5β-androstane-3α,17β-diol and reflects the loss of water from the molecular ion.

The CID spectra of 5α- and 5β-androstane-3α,11β-diol-17-one show markable difference in the lower fragments range: both of them (parent ion m/z 306) show the base peak m/z 270 which clue on two losses of water from the molecular ion and two other ions, m/z 288 and m/z 306 (Fig. 3). The loss of a third water in the CID spectrum of 5β-androstane-3α,11β-diol-17-one is seen clearly with the fragment m/z 252. But in the CID mass spectrum of 5α-androstane-3α,11β-diol-17-one the fragment m/z 252 is very poor and the fragment m/z 255 resulting from the loss of a methyl radical from the ion m/z 270 is more intensive than that of m/z 252.

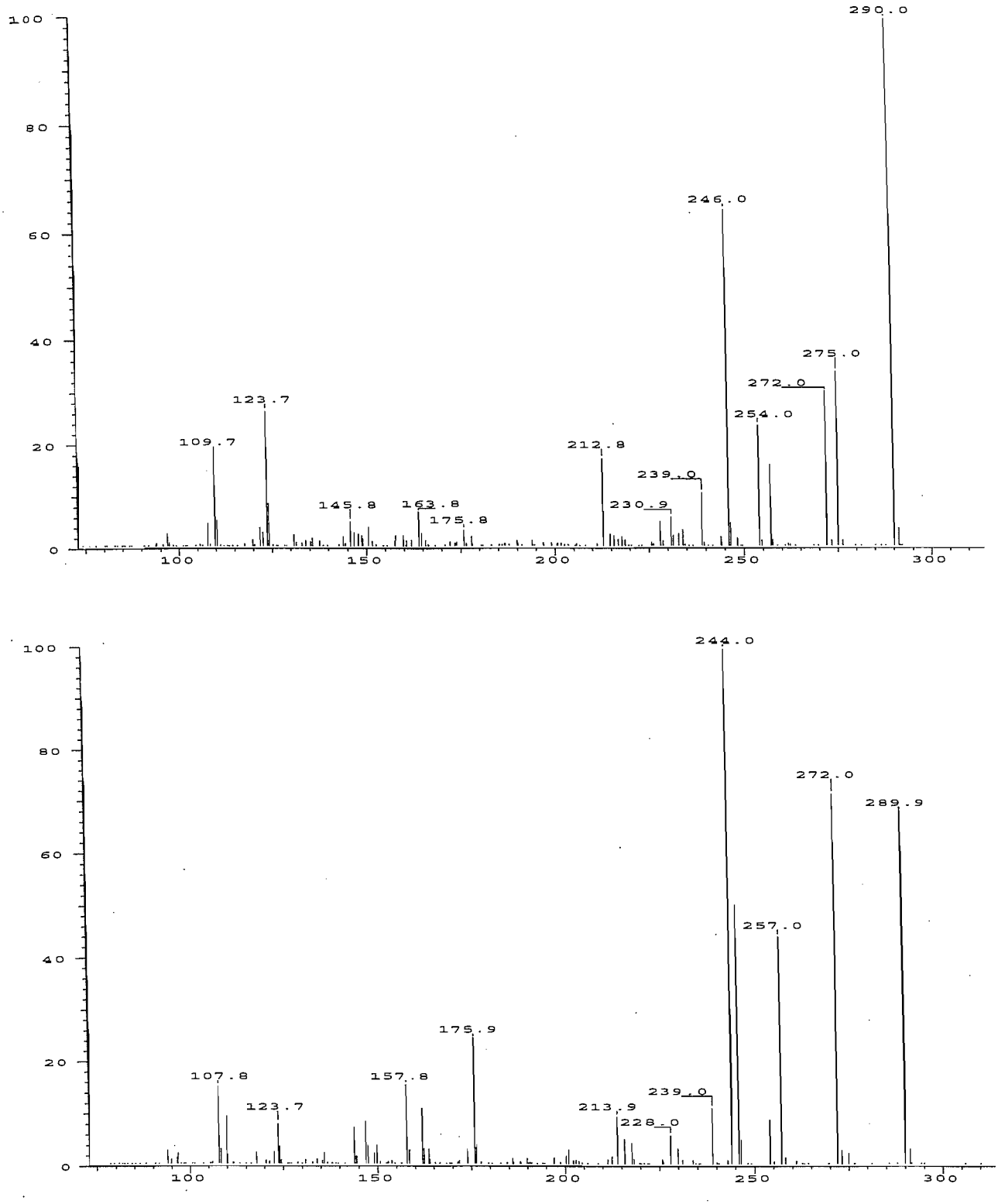

Fig. 1 The CID mass Spectra of Androsterone (up, 9.12 min) and Etiocholanolone (8.59 min)

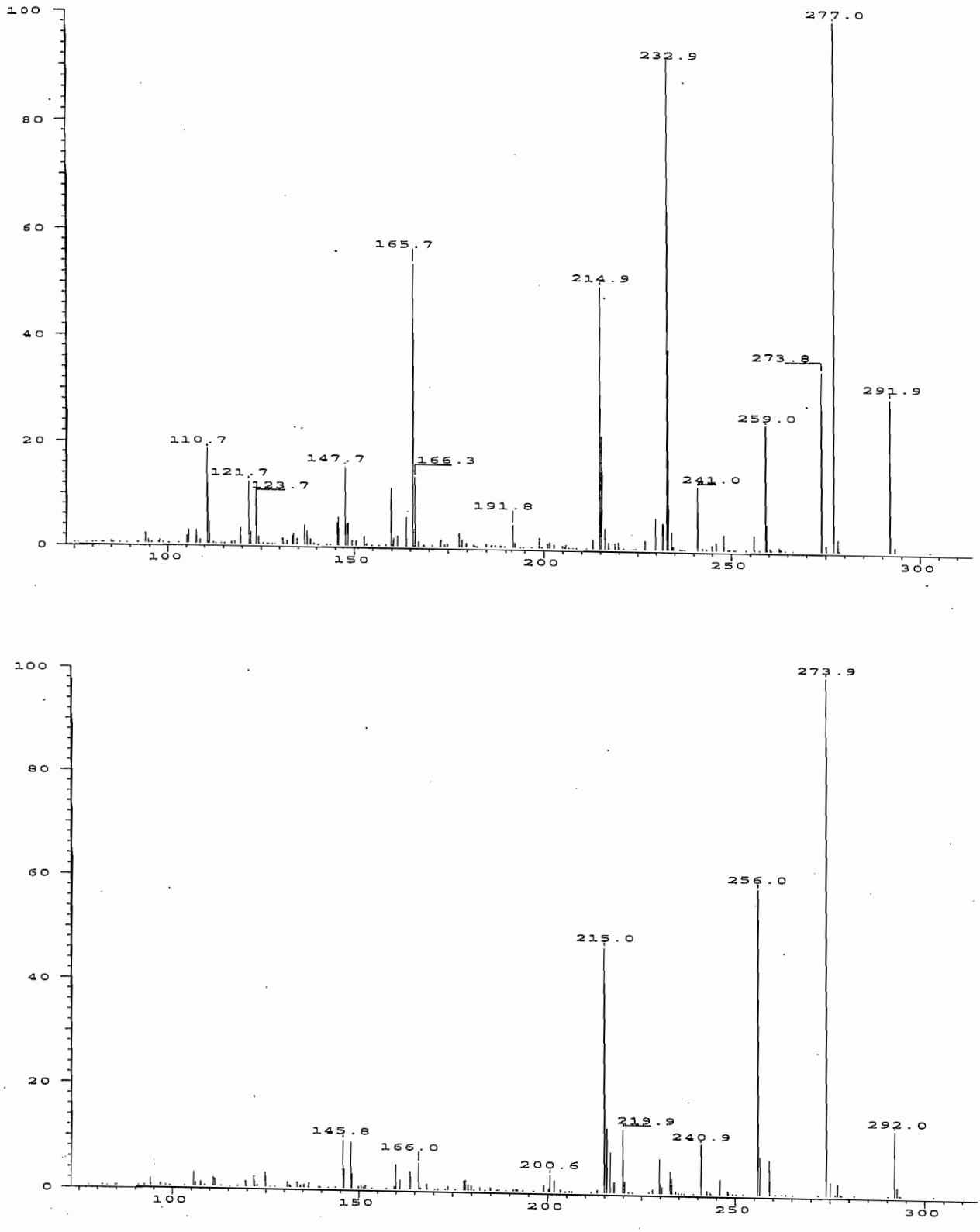

Fig. 2 The CID mass Spectra of 5α-Androstanediol (up, 9.28 min) and 5ß-Androstanediol (8.81 min)

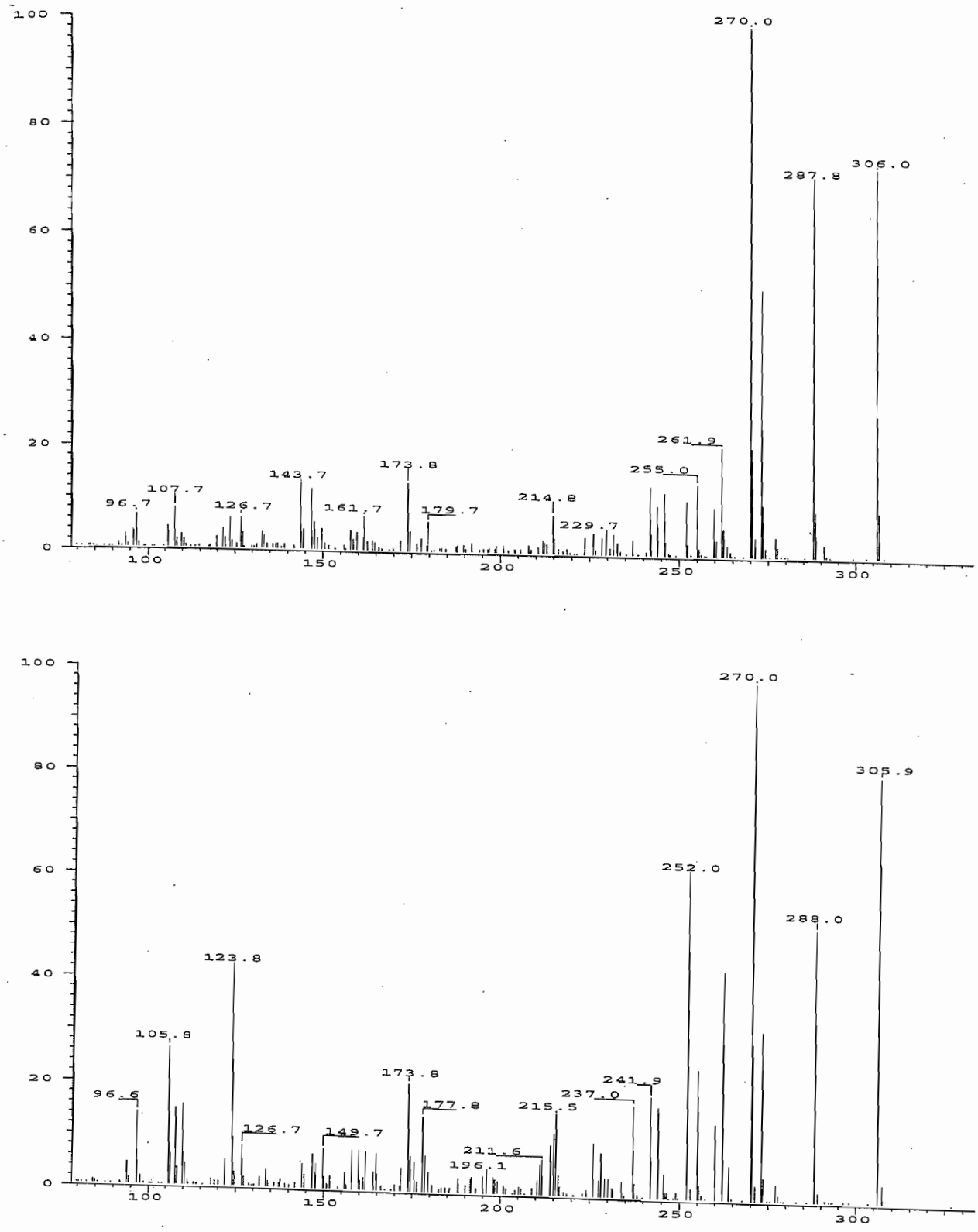

Fig. 3 CID mass Spectra of 11ß-HO-Androsterone (up, 12.49 min) and 11ß-HO-Etiocholanolon (11.66 min)

The different tendency to lose water or a methyl group from their parent ions is the clear evidence to differentiate the epimers by mass spectrometry . Fig. 4 shows that the trend of 5ß-androstan-3α-ol-17-one to lose water and the trend of 5α-androstan-3α-ol-17-one to lose a methyl group from the molecule are greatly increased by increasing the collision energy.

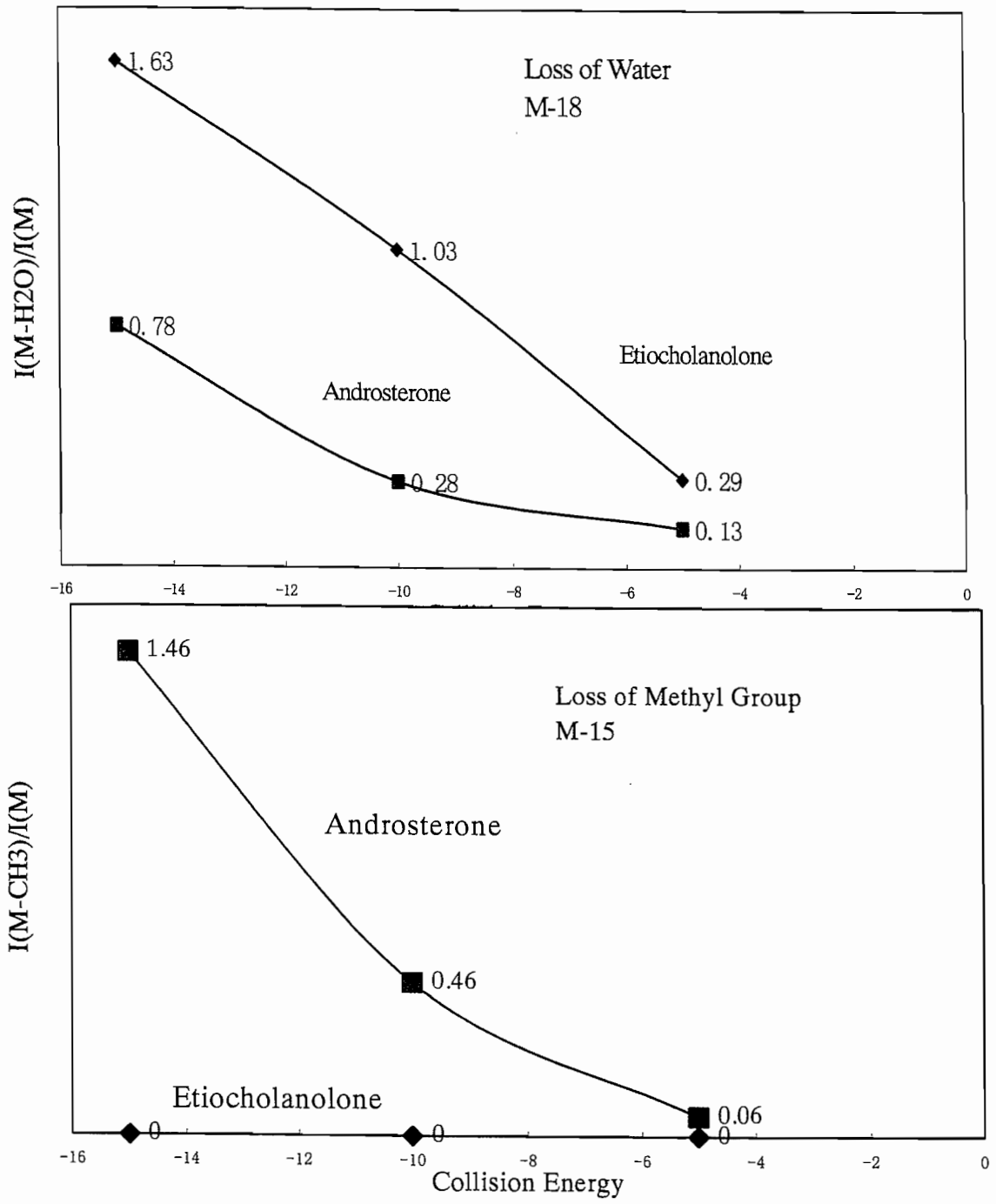

Fig. 4 The Tendency of Androstan-3α-ol-17-ones to lose Water or a Methyl Group with the Change of Collision Energy

The loss of water from 5β-androstane-3α,17β-diol increases with the increase of collision energy much stronger than that from 5α-androstane-3α,17β-diol. The intensity ratio of the ion m/z M^+-18 to the molecular ion M^+ of 5β-androstane-3α,17β-diol boosted from 2.03 to 16 when the collision energy was changed from –5 eV to –15 eV, while this ratio of 5α-androstane-3α, 17β-diol increased from 0.4 to 1.59 only. 5β-androstane-3α,17β-diol produces no clear fragment m/z 277 (M^+-15) under all tested conditions. Otherwise the loss of a methyl group from the 5α-androstane-3α,17β-diol molecule is greatly enhanced (see Fig. 5). The intensity ratio of the ion m/z M^+-15 to the molecular ion M^+ of 5α-androstane-3α,17β-diol increased 26 times.

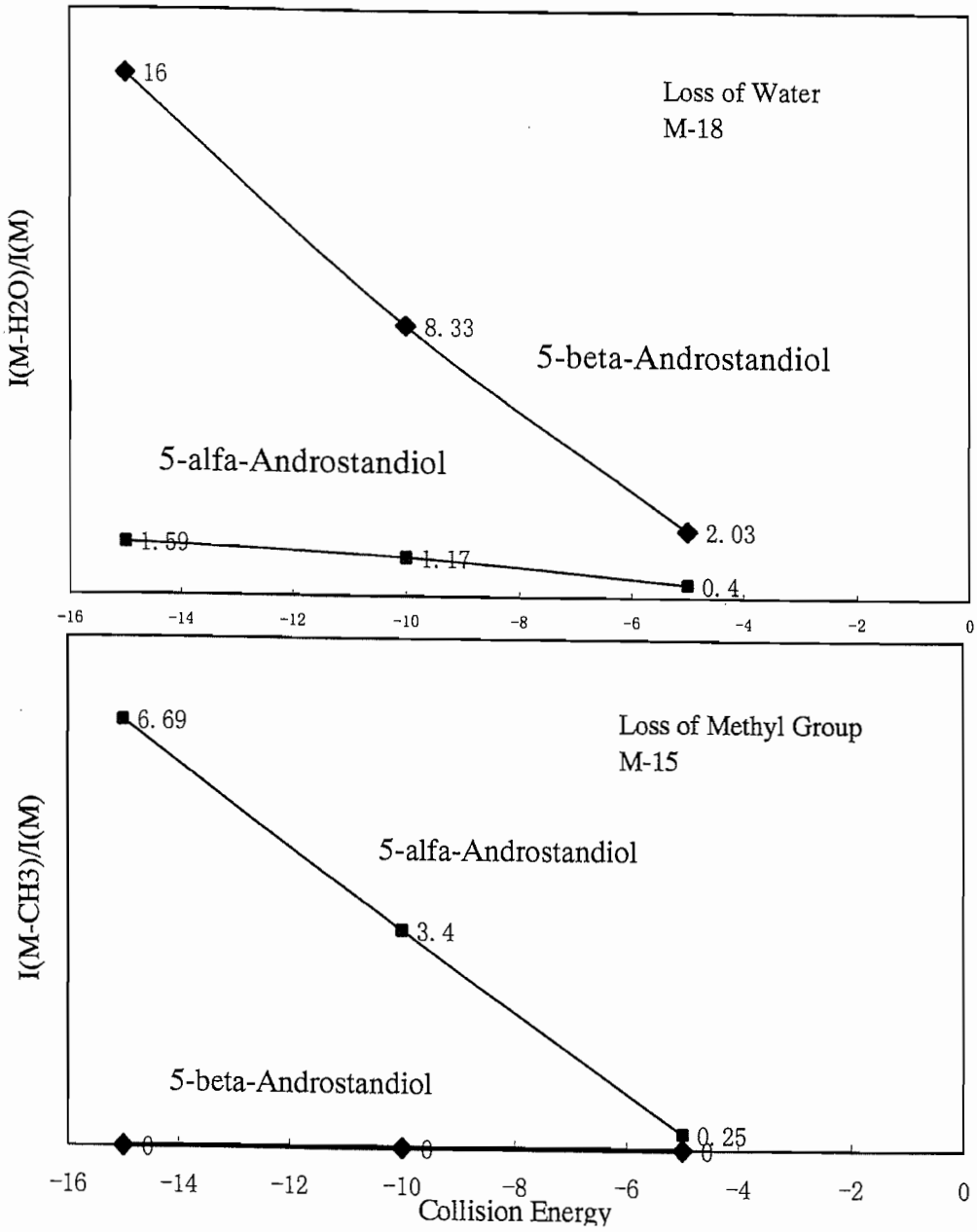

Fig. 5 The Tendency of Androstane-3α,17ß-diols to lose a Methyl Group or Water with the Change of Collision Energy

Neither 5α- nor 5β-androstane-3α,11β-diol-17-one show a clear fragment m/z 291, which would result from the loss of a methyl group from the molecular ion. In opposite to the two previous examples the loss of the first water from the 5α isomer seems to be easier than that from 5β-androstane-3α,11β-diol-17-one (due to the influence of the 11ß-hydroxy group ?). Both of them have similar behavior in the loss of the second water when the collision energy is increased. The intensity ratio of m/z M+-18-18 to M+ changed in parallel pattern (see Fig. 6)

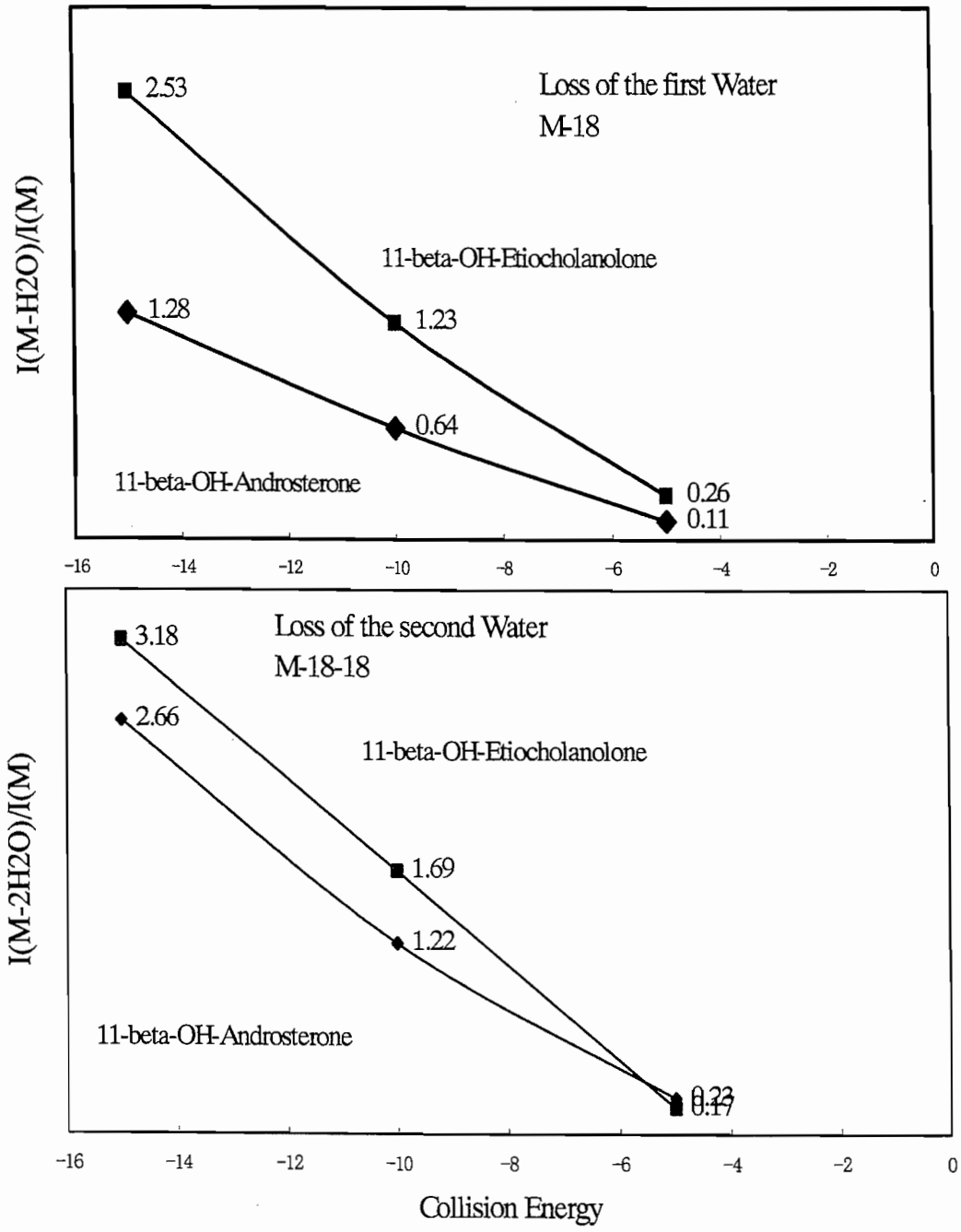

Fig. 6 The Tendency of Androstane-3α,11ß-diol-17-ones to lose the first and second Water

After the loss of two water both of them can lose a methyl group or the third water. In the CID spectra the fragment m/z 252 (M^+-18-18-18) of 5β-androstane-3α,11β-diol-17-one is much higher than that of 5α isomer. After the loss of the first water both lose a methyl group from the ion M^+-18 but with the increase of collision energy 5α-androstane- 3α,11β-diol-17-one shows a much larger intensity ratio (see Fig. 7).

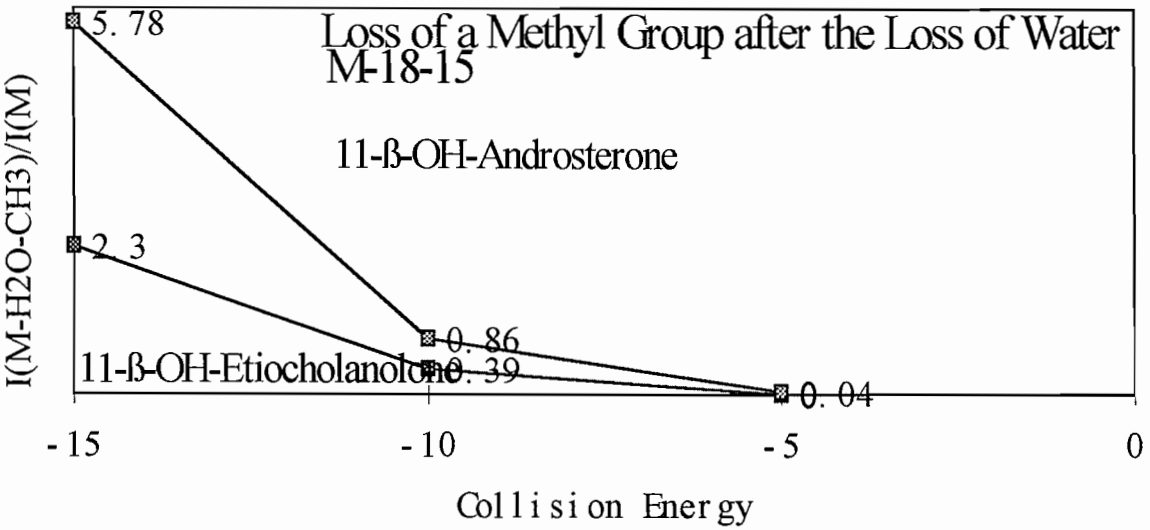

Fig. 7 The Tendency of Androstane-3α,11ß-diol-17-ones to lose a Methyl Group after the Loss of the first Water

After derivatization with MSTFA the CID spectra of 5α- and 5β-androstan-3α-ol-17-one, bis-*O*-TMS and of 5α- and 5β-androstane-3α,11β-diol-17-one, tris-*O*-TMS do not show any significant difference though the collision energy is changed in a great range. But the androstane-3α,17β-diols, bis-*O*-TMS are an exception. 5α- and 5β-androstane-3α,17β-diol, bis-*O*-TMS can be distinguished by their CID spectra (also molecular ion as parent ion). Fig. 8 shows their CID spectra (collision energy –10 eV).

While the 5ß-isomer shows up m/z 346 and m/z 256 (the latter as base peak resulting from two losses of trimethylsilanol) and in low intensitity m/z 241 (resulting from the loss of a methyl radical from m/z 256) the 5α-isomer shows up also the fragments m/z 421, 331 and 241 (the latter as base peak) all generated by the loss of a methyl radical from the relevant parent ions m/z 436, 346 and 256. Hereby the strong tendency of the 5α-isomer to lose a methyl group as demonstrated in Fig. 5 is confirmed.

In a case due to a huge amount of interfere the retention times of 5α-androstane-3α,17β-diol, bis-*O*-TMS and others shifted and the peak of 5α-androstane-3α,17β-diol, bis-*O*-TMS showed only a small sidehill in the tail the interfering peak. With the CID spectra 5α- and 5β-

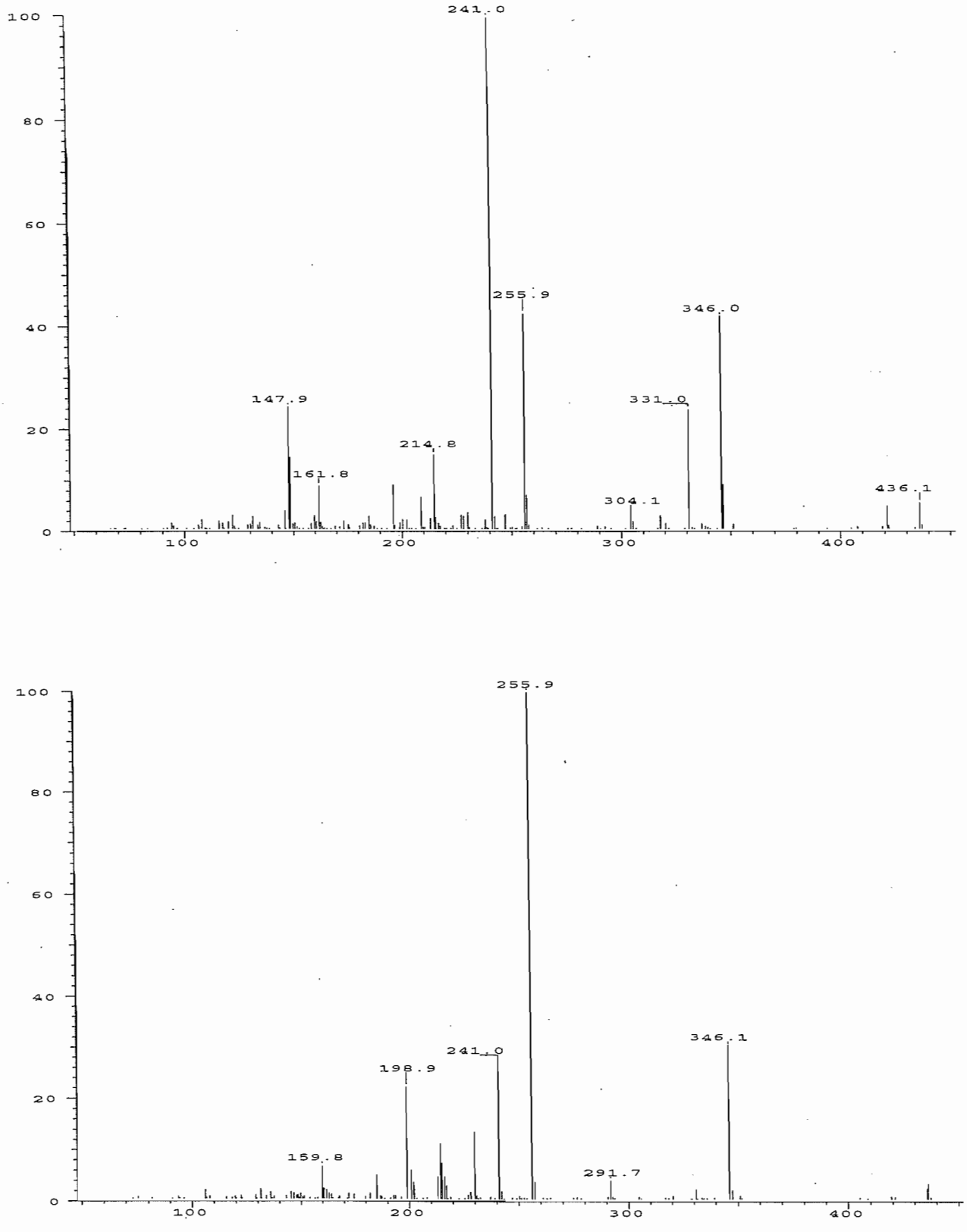

Fig. 8 The CID Spectra of 5α-Androstanediol, bis-TMS (up, 11.24 min) and 5ß-Androstanediol, bis-TMS (11.38 min)

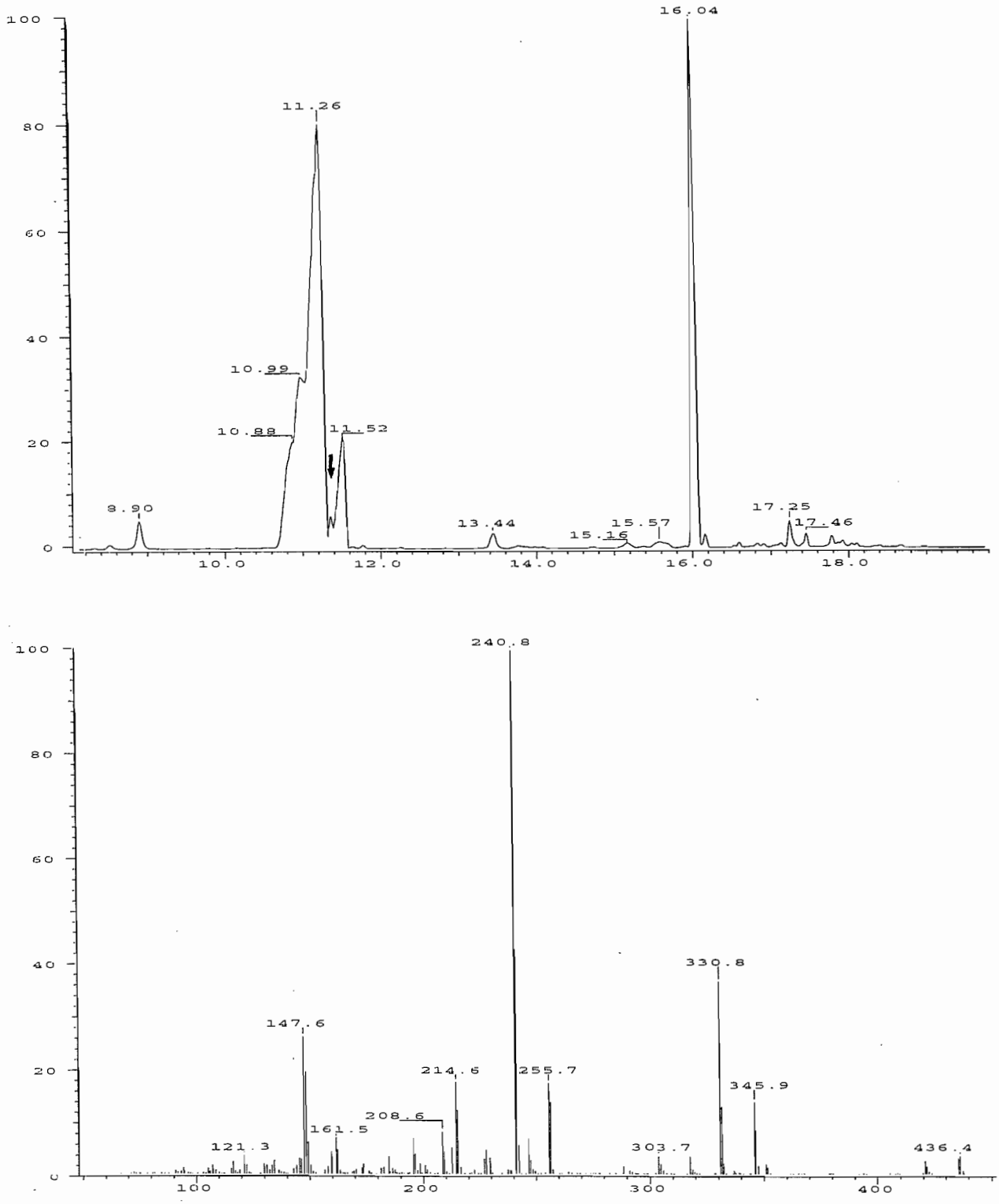

Fig. 9 RIC (m/z 436 parent ion , daughter ion mode) and CID mass Spectrum of the Peak at 11.37 min

androstane-3α,17β-diol could be confirmed and the ratio of them could be estimated. Fig. 9 shows the chromatogram (m/z 436 as parent ion, daughter ion mode) and the mass spectrum of the peak with the retention time of 11.37 min between two huge peaks.

Conclusion

1) Without derivatization, the CID mass spectra of the 5α- and 5β-isomers of some anabolic androgenic steroids (AAS) tested in this study show significant difference, which may distinguish and confirm these substances.

2) After derivatization with MSTFA only 5α and 5β-androstane-3α,17β-diol, bis-*O*-TMS can be distinguished by the CID mass spectra.

3) In one case, the retention time of 5α-androstane-3α,17β-diol, bis-*O*-TMS was shifted due to the presence of a huge amount of an interfering peak. In this case 5α-androstanediol and 5β -androstanediol were confirmed by the CID spectra.

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