JOURNAL OF MASS SPECTROMETRY
*J. Mass Spectrom.* **33**, 872–883 (1998)

# Energy-resolved Collision-induced Dissociation Atmospheric Pressure Chemical Ionization Mass Spectrometry of Constitutional and Stereo Steroid Isomers

**Per J. R. Sjöberg and Karin E. Markides***
Department of Analytical Chemistry, Uppsala University, P.O. Box 531, S-751 21 Uppsala, Sweden

**Energy-resolved mass spectra were recorded by controlled up-front collision-induced dissociation (CID) or conventional CID tandem mass spectrometry. Differentiation between constitutional isomers and stereoisomers of hydroxysteroids was studied using atmospheric pressure chemical ionization interfaced to a triple quadrupole mass spectrometer. Relative abundances of fragment ions from mainly water loss were used. © 1998 John Wiley & Sons, Ltd.**

KEYWORDS: energy-resolved mass spectrometry; atmospheric pressure chemical ionization; collision-induced tandem mass spectrometry; up-front collision-induced dissociation; steroids

## INTRODUCTION

Steroids containing oxygenated substituents are frequently present in natural products[1] and it is well known that their physiological activities are influenced by the stereochemistry of the respective substituents. In order to study this relationship, reliable methods are necessary for determining the configuration of the functional groups. The mass spectrometric behaviour of steroids was intensively studied over 20 years ago[2] and its relationship to the stereochemistry of the steroids has recently been reviewed.[3] Most of these studies were performed with hard ionization techniques that give a high degree of fragmentation and that were commonly used at the time. Sector instruments have, for example, been used in combination with mass analysed ion kinetic energy (MIKE) scanning techniques[4] for the investigation of metastable ion dissociation in order to obtain information about the stereochemistry involved. Low-pressure sources such as electron ionization (EI),[2] chemical ionization (CI)[5] and fast atom bombardment (FAB)[6] have today been replaced by the increasingly popular mild and efficient ion sources operating at atmospheric pressure.[7–9]

It is known that collision-induced dissociation (CID) of low kinetic energy ions[10] in a triple-quadrupole mass spectrometer[11,12] can be used to obtain structural information about the precursor ion. The CID can be performed in two ways ['up-front' CID or tandem mass spectrometry (MS/MS)] in a triple-quadrupole mass spectrometer with an ion source operating at atmospheric pressure.[13–17]

The atmospheric pressure chemical ionization (APCI) technique will, in the positive-ion mode, mainly generate molecular ions $[M]^{+\cdot}$ or quasi-molecular ions $[M + H]^+$, and only a few fragment ions.[18,19] Therefore, it is normally impossible to differentiate between constitutional or stereoisomers of steroids by APCI-MS spectrometry. Fixed-energy APCI-MS/MS might, however, reveal important structural information about a precursor ion, but much more information can be obtained simply by monitoring the abundances of the dissociation products as a function of the energy of the precursor ion. This technique has previously been referred to as energy-resolved atmospheric pressure chemical ionization mass spectrometry (API-ERMS).[20]

API-ERMS using APCI was initially used by Kambara *et al.*[21] for the differentiation of isobaric cluster ions based on their dissociation energies. Furthermore, APCI-ERMS has also been used to characterize the dissociation process and to determine relative bond energies, making it possible to predict dissociation pathways of a series of homologuous organophosphorus compounds.[20,22] To our knowledge, APCI-ERMS has not been used before for the differentiation of steroid isomers.

The main purpose of this work was to demonstrate the possibility of distinguishing between hydroxysteroid isomers and stereoisomers by the use of APCI in combination with CID in different regions of a triple-quadrupole mass spectrometer. Furthermore, different analyte introduction methods were utilized in order to

* Correspondence to: P. J. R. Sjöberg, Department of Analytical Chemistry, Uppsala University, P.O. Box 531, S-751 21 Uppsala, Sweden.

Contract/grant sponsor: Swedish Natural Research Council; Contract/grant number: K-1439-318.

CCC 1076–5174/98/090872–12 $17.50


*Received 11 February 1997*
*Accepted 8 June 1998*

investigate the influence of cluster ion composition on the spectra obtained.

## EXPERIMENTAL

### Chemicals

The analytes used in this study were digoxigenin (**1**), gitoxigenin (**2**), 5α-androstane-3α,17β-diol (**3**), 5α-androstane-3β,17β-diol (**4**), 5β-androstane-3α,17β-diol (**5**) and 5β-androstane-3β,17β-diol (**6**) (Sigma Chemical, St Louis, MO, USA), 6β,11β,21-trihydroxy-4-pregnene-3,20-dione (**7**) (6-hydroxycorticosterone) (Steraloids, Wilton, NH, USA) and 11β,17α,21-trihydroxy-4-pregnene-3,20-dione (**8**) (hydrocortisone) (Upjohn, Kalamazoo, MI, USA).

Methanol and acetonitrile of LC gradient grade were supplied by Merck (Darmstadt, Germany) and used without further purification. Water was obtained from a Milli-Q Plus purification system (Millipore, Bedford, MA, USA).

### Sample introduction

The analytes were introduced using continuous slow flow supercritical fluid extraction (SFE) or liquid infusion through a micro-heated nebulizer interface.

For the SFE introduction, an interface previously developed in-house was used.[23,24] The analytes were dissolved in methanol (0.5–1.0 mg $ml^{-1}$) and 10–15 μl of the solution were injected into an LC precolumn (30 × 4 mm i.d., packed with 5-μm $C_{18}$ $SiO_2$ particles) connected to a Lee Scientific Series 600 SFC pump (Dionex, Sunnyvale, CA, USA). Before extraction, the solvent was evaporated using a 50 atm inlet $CO_2$ pressure and an unrestricted outlet. A 1 m × 50 μm i.d. × 190 μm o.d. fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) was then connected to the LC precolumn and an integral restrictor was made as described earlier.[23] Finally, the $CO_2$ pressure was increased to 200–300 atm and the SFC/GC oven (Lee Scientific Series 600) temperature was set to 40 °C.

A micro-heated nebulizer interface constructed in-house[25] was used for introduction of the analytes in solvent mixtures. Flow injections were made with a C6W injection valve, (Valco Instruments, Houston, TX, USA), equipped with a 15 μl sample loop. The analytes were dissolved in 50:50 (v/v) methanol–water or acetonitrile–water and infused through a 1.2 m × 15 μm i.d. × 145 μm o.d. fused-silica capillary (Polymicro Technologies) at a flow-rate of 0.3 μl $min^{-1}$ using a PU 980 LC pump (Jasco, Tokyo, Japan) operated at constant pressure. The 15 μm i.d. fused-silica capillary tip was positioned ~ 0.5 mm outside the 250 μm i.d. fused-silica nebulization capillary, through which there was a flow of 0.3 l $min^{-1}$ of nebulization gas (air of FID quality; AGA, Stockholm, Sweden). Co-axial with the nebulization capillary there was a larger (700 μm i.d. × 850 μm o.d.) fused-silica capillary, which held a heating wire coil to which a controlled voltage could be applied with a laboratory-made device in order to vaporize the liquid mobile phase prior to the ionization. The nebulization was aided by an additional gas flow (0.5 l $min^{-1}$ of air of FID quality, AGA) between the nebulizer capillary and the larger fused-silica capillary.

### MS conditions

An API III triple-quadrupole mass spectrometer (Perkin-Elmer SCIEX, Concord, ON, Canada) was upgraded to an API $III^+$ instrument in the course of the study; either instrument was equipped with a point to plane corona discharge ion source. The discharge current was 5 μA and the potential was set to 650 V at the interface plate and 30 V at the first focusing r.f.-only quadrupole (Q0). For the MS/MS experiment the orifice potential (OR) was set to 35 V. The volumetric flow-rate of the counter-current dry nitrogen curtain gas, 99.9999% purity (6.0 AGA), was 1.0 l $min^{-1}$ over the sampling orifice and it was heated to 50 °C. The collision gas was argon, 99.9999% pure (6.0 AGA). Data were acquired either by scanning Q1 or Q3 in increments of 0.1–0.2 u with a dwell time of 1–2 ms. In some experiments selected ion monitoring (SIM) was performed with a dwell time of 50 ms. ERMS was performed in MS and MS/MS modes. In the MS mode, (i.e. 'up-front' CID), the kinetic energy of the cluster ions was increased by linearly increasing the orifice potential in 1 V increments while keeping the first focusing r.f.-only quadrupole (Q0) at a fixed potential. The potential difference between OR and Q0 is presented in the text as the drift potential and determines the collision energy in 'up-front' collision. In the MS/MS mode, 10 spectra were recorded in the multi-channel acquisition (MCA) mode at each energy level in steps of 2–10 V. As indicated by the instrument software, the collision gas thickness was $300 \times 10^{12}$ molecules $cm^{-2}$ when the API III was used and $200 \times 10^{13}$ molecules $cm^{-2}$ for the API $III^+$, unless stated otherwise. The potential difference between Q0 and the collision cell (Q2) determines the MS/MS collision energy. Furthermore, collision gas thickness resolved mass spectrometry (CGTR-MS) was performed using a collision energy of 50 eV.

## RESULTS AND DISCUSSION

In the triple-quadrupole instrument used in this study, the molecular, quasi-molecular or cluster ions that are generated in the ion source are first drawn through the interface plate opening (2 mm diameter) into the dry nitrogen plenum region and further on through a sampling orifice (0.110 mm diameter) and, by an expansion, into the high vacuum of the mass spectrometer. In the high vacuum the mean free path of the ions increases rapidly with the distance from the orifice and ions gain axial kinetic energy from the applied electric fields. By manipulating the potential difference between the sampling orifice (OR) and the first focusing r.f.-only quadrupole (Q0), the axial kinetic energy of the ions can be varied, and collisions between ions and neutral nitrogen

molecules thus generate 'up-front' CID. In addition, CID can of course also be performed by MS/MS in a central quadrupole collision cell (Q2), were efficient fragmentation can be achieved.[26] By manipulation of the potential difference between the first r.f.-only quadrupole (Q0) and the collision cell (Q2), the axial kinetic energy of a precursor ion can be controlled.

An example of differentiation of constitutional steroid isomers can be seen in Fig. 1, where the CID MS/MS energy was varied between 0 and 90 eV in both positive and negative modes. The base peak selected for CID MS/MS was the protonated molecule $[M + H]^+$ in the positive mode and the analyte adduct ion $[M + O_2]^-$ (from the superoxide anion $O_2^-$) in the negative mode.[27,28] It can easily be seen in Fig. 1 that the loss of water from gitoxigenin is strongly favoured compared with that from digoxigenin. Studying the structural differences of the two compounds chosen here (see Fig. 1), it can be argued that this behaviour is due to the formation of a conjugated double bond system; an in-depth study of the true mechanism would, however, need a more narrowly defined system. Similar results have been reported previously by Bruins[29] for negative ion desorption chemical ionization of the cardiac glycosides digoxin and gitoxin.

It is known that different cluster ions, i.e. $[M + H + H_2O]^+$, $[M + H + CH_3OH]^+$ and $[M + H + CH_3CN]^+$, are formed as base peaks depending on the type of interface and solvent used for sample introduction. CID MS/MS experiments were performed on androstanediols to study the influence of cluster ion selection on the relative abundances of fragment ions.

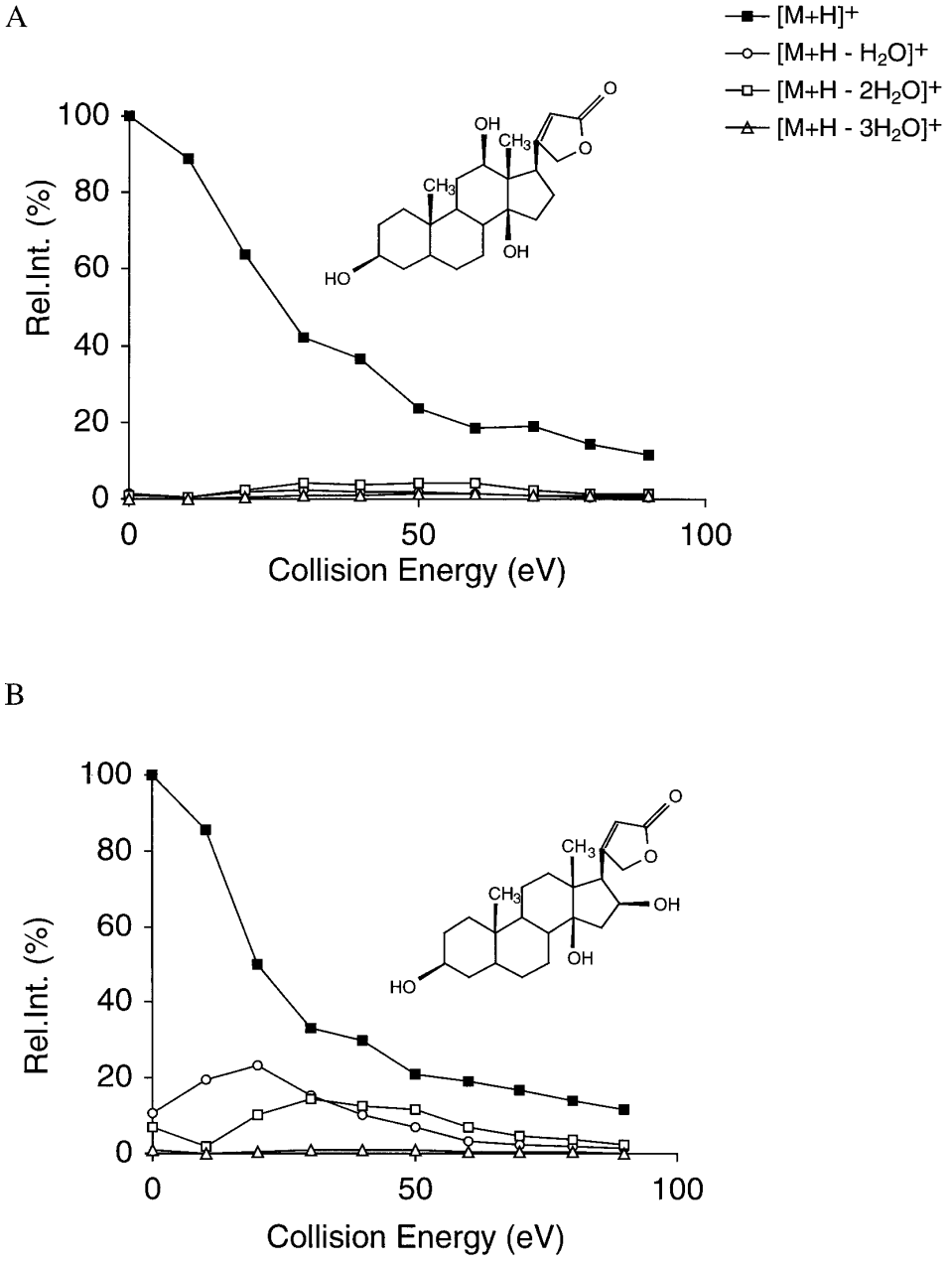
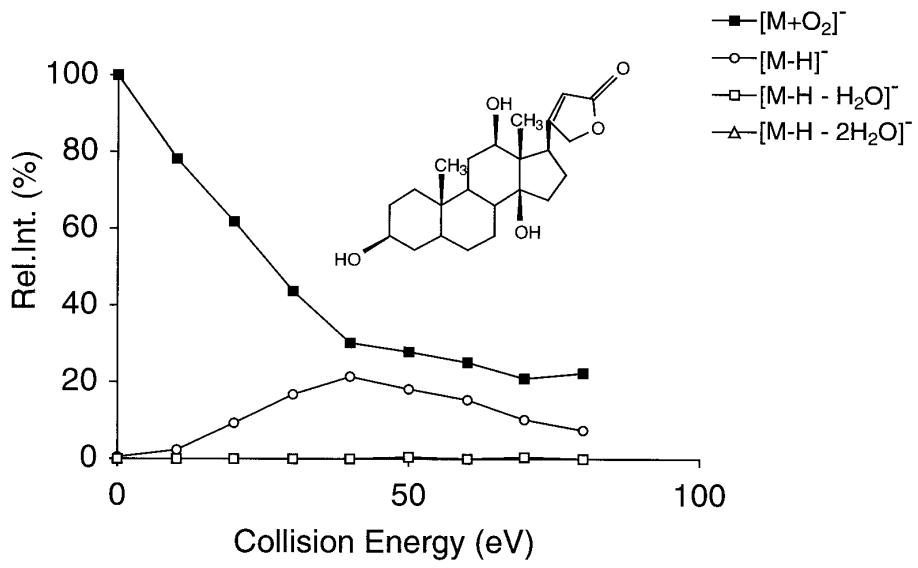



**Figure 1.** Continuous slow flow SFE of (a) digoxigenin (**1**) and (b) gitoxigenin (**2**) in the positive ionization mode and (c) digoxigenin (**1**) and (d) gitoxigenin (**2**) in the negative ionization mode.

C




D

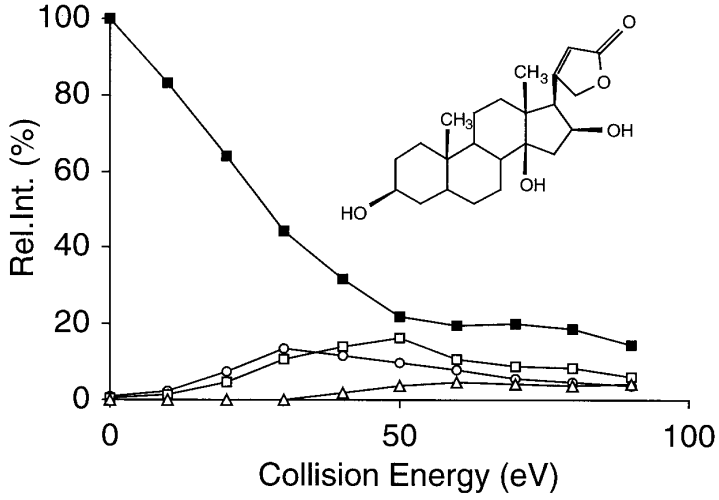



**Figure 1.** *Continued*

The first system studied was a continuous slow flow SFE using neat $CO_2$ as carrier and with water-moistened air as nebulizer gas. The $[M + H + H_2O]^+$ cluster ions were predominantly formed in this system and selected as parent ions for CID MS/MS. As can be seen in Fig. 2, it was then possible to detect differences in the degree of water loss between the stereoisomers **3** and **4**.

The abundance of the $[M + H - H_2O]^+$ ion is slightly lower and that of the $[M + H - 2H_2O]^+$ ion is greater for **3** than **4**. The experiments were repeated several times and the relative abundance is plotted *vs.* the collision energy in Fig. 3. An explanation for this observation could be that the $[M + H - H_2O]^+$ ion generated from **3** is more unstable and that this will result in a higher abundance of the $[M + H - 2H_2O]^+$ ion. Different degrees of dehydration for the isomers were also seen by manipulation of the collision gas thickness (CGT) using a low-pressure collision cell. When the MS instrument was upgraded to a high-pressure collision cell,[30,31] a more efficient fragmentation resulted and the same differences could still be seen but at slightly lower collision energies (Fig. 4).

Differences in degree of water loss could be observed from **3** and **4** when CI with methane as reactant gas was used.[32,33] Promé *et al.*[33] used $NH_3$ CI, and observed almost no difference in the $[M + H - 2H_2O]^+/[M + NH_4]^+$ ion abundance ratio for androstane-3,17$\beta$-diols. However, small differences in $[M + H - 2H_2O]^+/[M + H - H_2O]^+$ ion abundance ratio could be observed. It was found in this study that the cluster ions formed when different solvents are used for

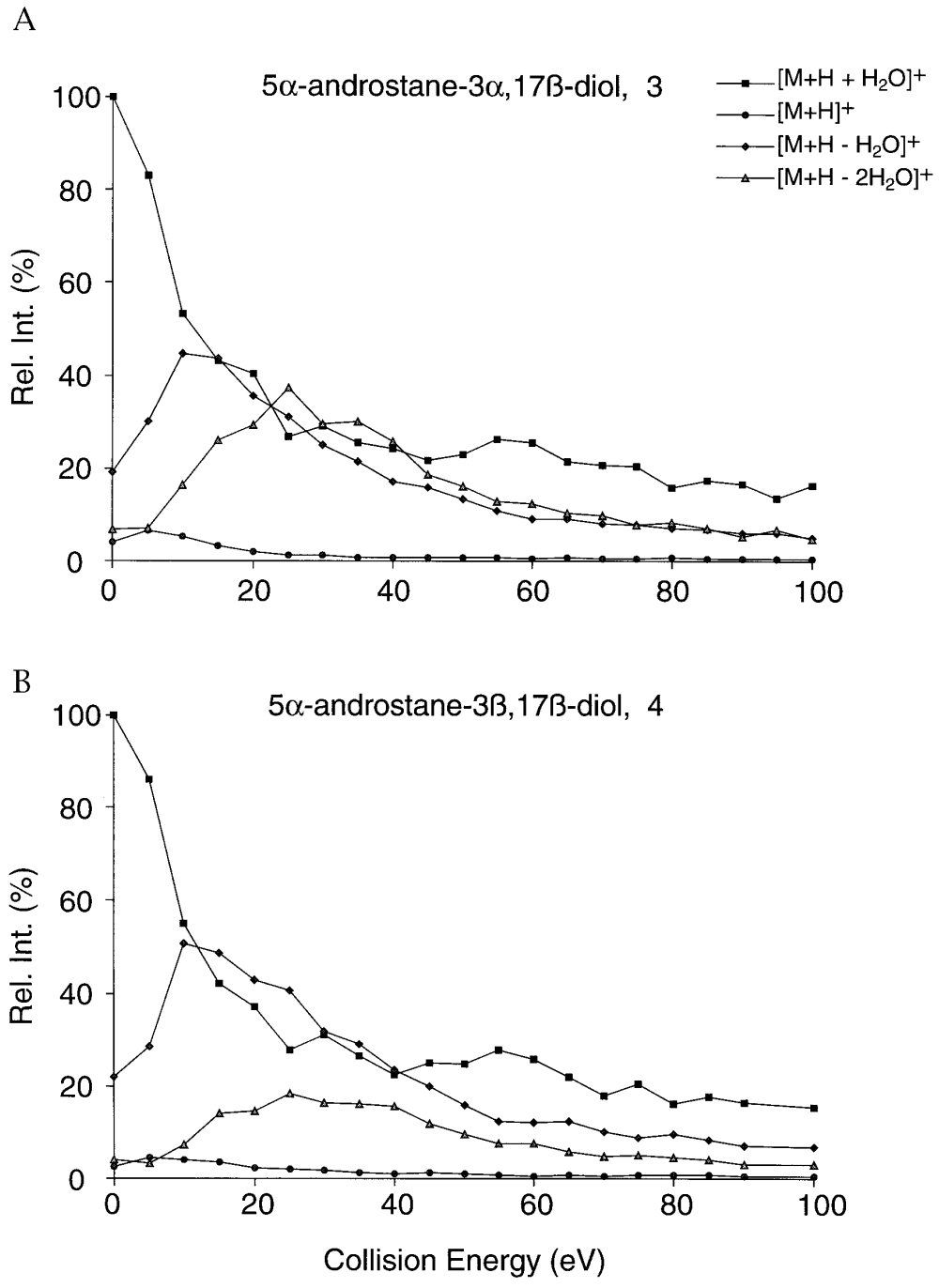



**Figure 2.** Energy-resolved spectra of 5α-androstane-3,17β-diols **3** and **4**. Continuous slow flow SFE was used for analyte introduction and the parent ion $[M + H + H_2O]^+$ was selected for CID MS/MS with a collision gas thickness of $300 \times 10^{12}$ molecules $cm^{-2}$.

analyte introduction with the micro- heated nebulizer could to some extent be utilized to differentiate between stereochemical variations. Figure 5(A) and (B) show that it was possible to differentiate between **3** and **4** when CID MS/MS was performed with a high-pressure collision cell using the methanol cluster ions as precursor ion. No clear differences in stereochemistry between **4**, **5** and **6** could be detected [see Fig. 4 (B)–(D)]. Similar results were achieved with the formation of acetonitrile cluster ions.

Stereospecific loss of water is the typical dissociation of hydroxysteroids and the analysis of experimental results must be performed carefully because thermal dehydration of neutral alcohols prior to ionization can also be involved.[2] As can be seen in Fig. 6, small signals from $[M + H - H_2O]^+$ and $[M + H - 2H_2O]^+$ ions are present in the up-front CID spectra which could possibly be caused by a minor degree of thermal dehydration in the ion source. To minimize thermal dehydration, an alternative ionization method to APCI, electrospray, could be used. However, essentially the same energy-resolved mass spectra were obtained in the MS/MS mode, which indicates that thermal dehydration does not affect the results when the heat applied to

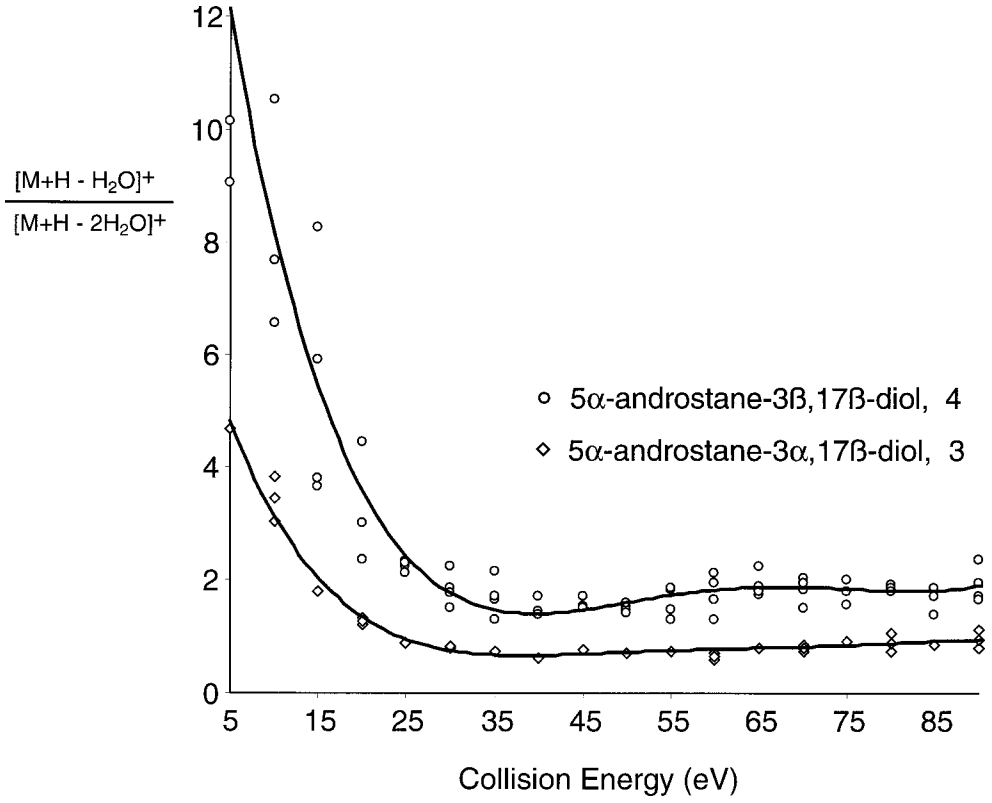



**Figure 3.** Relative abundance, $[M + H - H_2O]^+/[M + H - 2H_2O]^+$, plotted *vs*. the collision energy for **3** and **4**. For experimental conditions, see Fig. 2.

the nebulizer is adjusted carefully. Furthermore, the soft nature of APCI due to the short length of the mean free path of the ions and frequent low-energy collisions in the interface will also contribute to the low dehydration seen in the interface when the normal (Q1) scan mode is used. Using 'up-front' CID demonstrates the possibility of differentiating between steroid isomers using a single-quadrupole APCI-based mass spectrometer.

Finally, selection of the ionization mode is an important parameter and can in some cases have a strong influence on the possibility of differentiating between isomers. An example is shown in Fig. 7, where the base peak $[M + H]^+$ or $[M^+O_2]^-$, depending on ionization mode, was selected for CID. The negative ionization mode gives larger differences in the tandem mass spectra between 6$\beta$,11$\beta$,21-trihydroxy-4-pregnene-3,20-dione and 11$\beta$,17$\alpha$,21-trihydroxy-4-pregnene-3,20-dione. The $[M + O_2]^-$ adduct ion was only observed in this study for steroids containing a carbonyl group.

## CONCLUSION

This study has shown that it is possible to differentiate between some hydroxysteroid isomers by monitoring the loss of water as a function of collision energy using soft APCI with a quadrupole instrument in either the MS or MS/MS mode.

### Acknowledgements

The digoxigenin and gitoxigenin compounds were kindly donated by Dr Steven W. Graves, Brigham and Women's Hospital, MA, USA. Financial support from the Swedish Natural Research Council, project K-1439-318, is gratefully acknowledged.

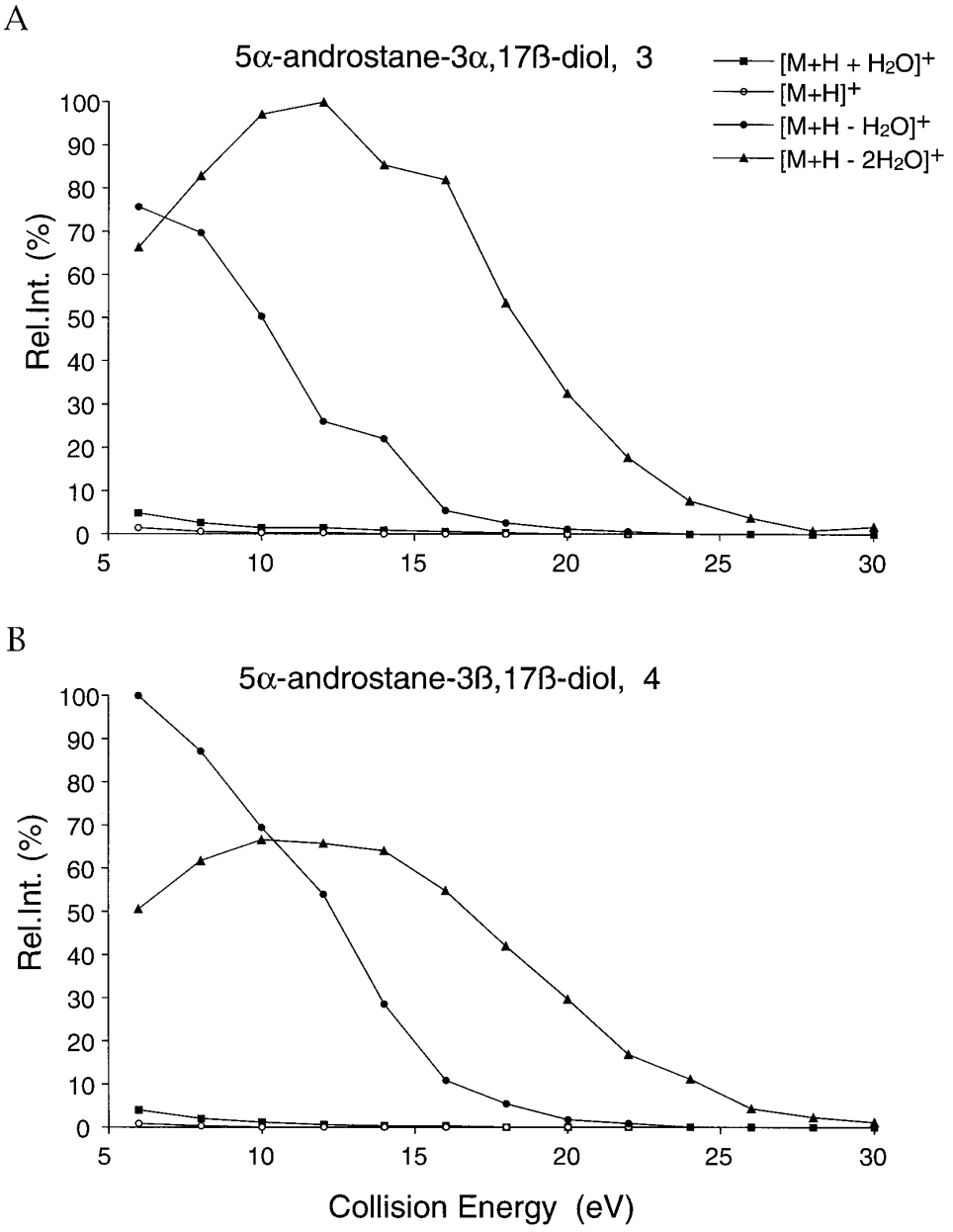



**Figure 4.** Energy-resolved spectra of 5*α*-androstane-3,17*β*-diols **3** and **4**. SFE was used for analyte introduction and the parent ion $[M + H + H_2O]^+$ was selected for CID MS/MS using an API III$^+$ instrument with a collision gas thickness of $200 \times 10^{13}$ molecules cm$^{-2}$.

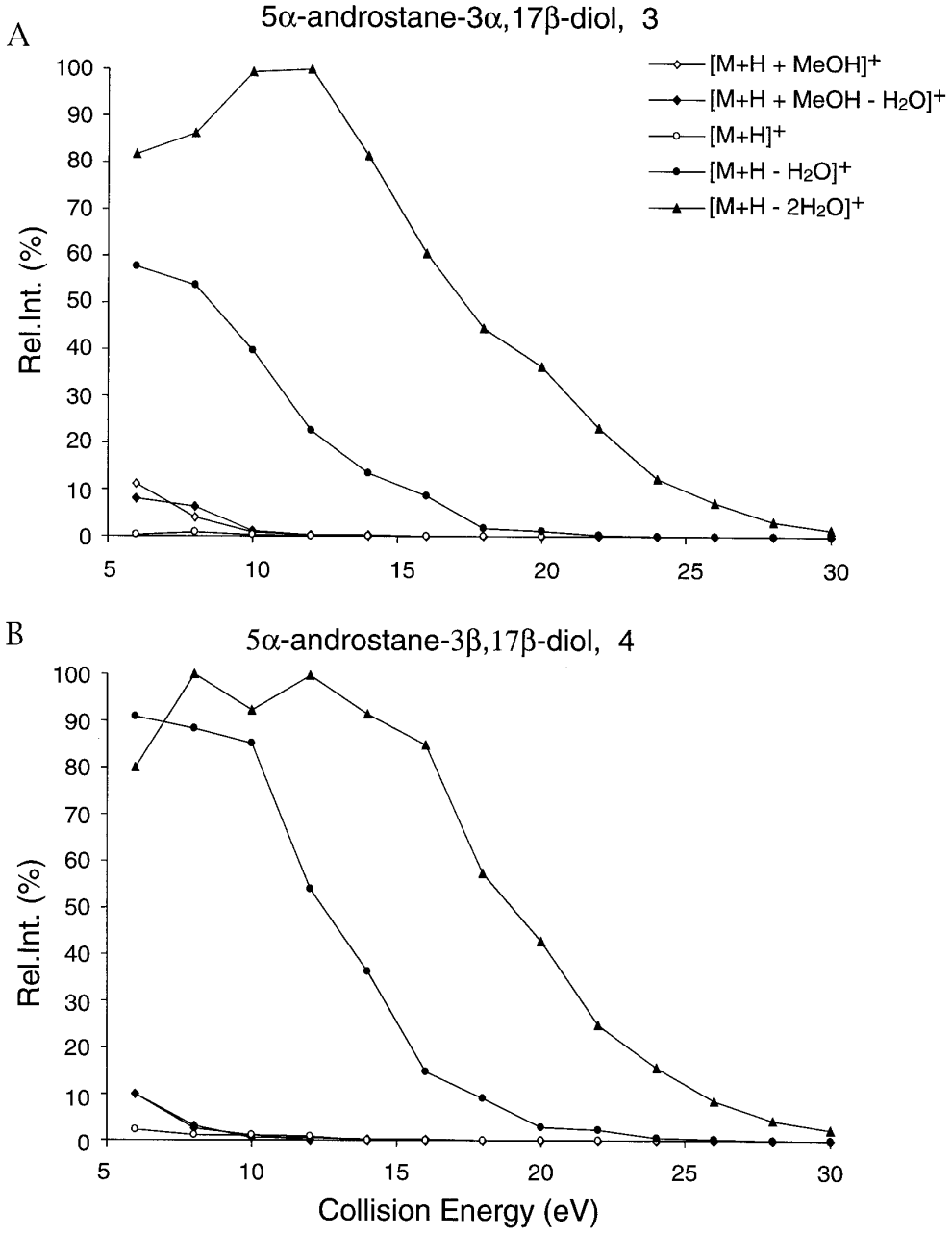



**Figure 5.** Energy-resolved spectra of androstane-3,17$\beta$-diols **3**–**6**. The analytes were dissolved in methanol–water (50:50) and introduced using a micro-heated nebulizer interface. The parent ion $[M + H + MeOH]^+$ was selected for CID MS/MS using an API III$^+$ instrument with a collision gas thickness of $200 \times 10^{13}$ molecules cm$^{-2}$.

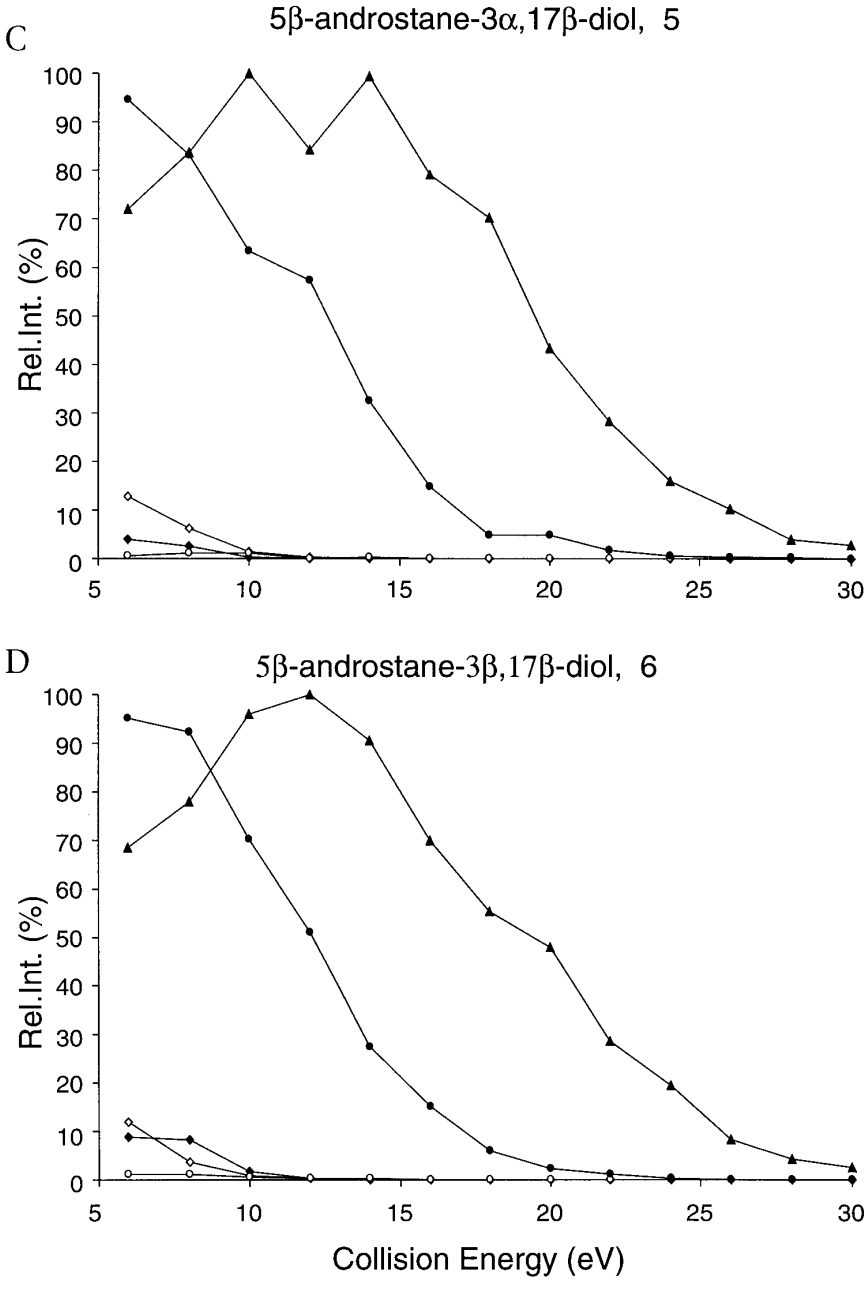



**Figure 5.** *Continued*

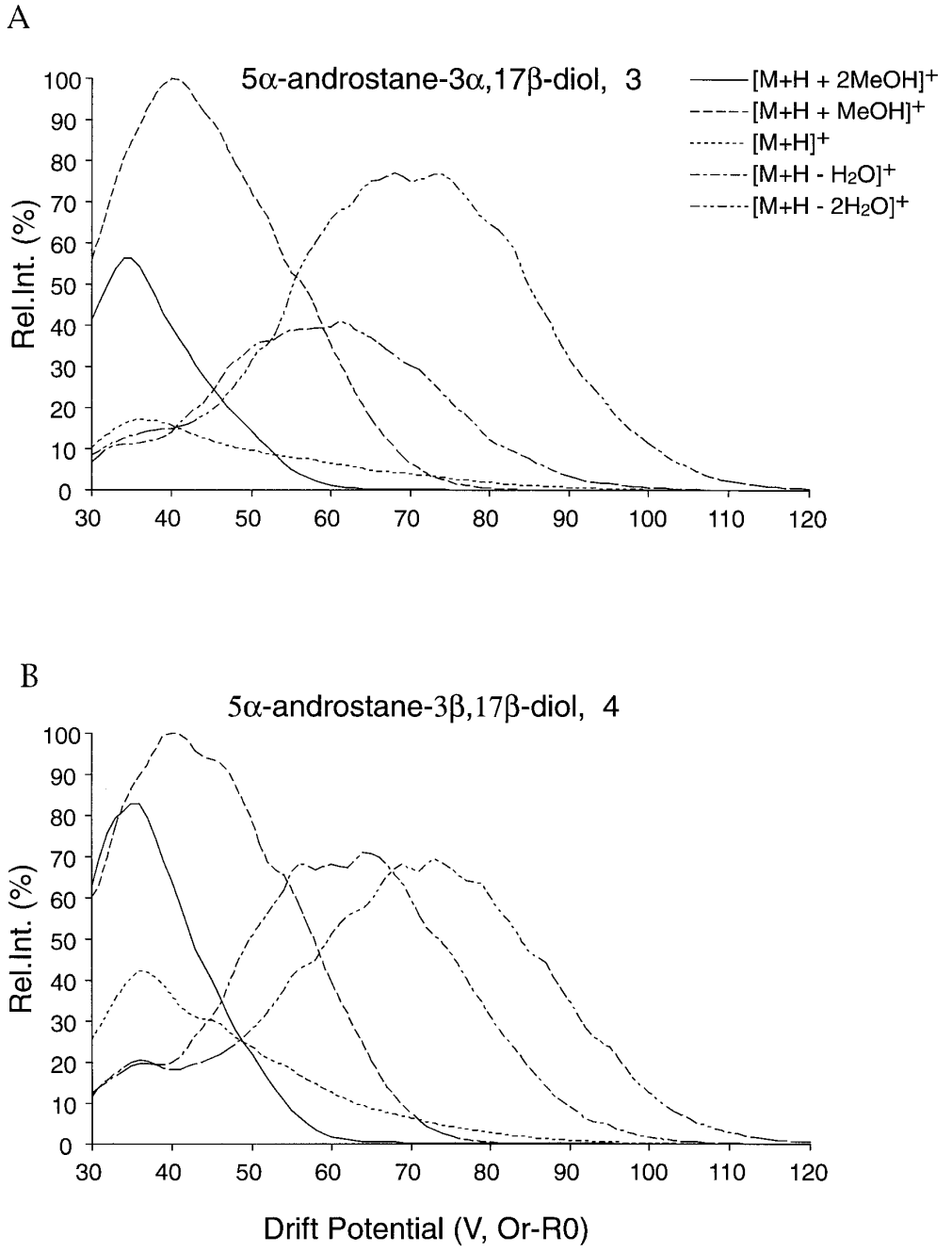



**Figure 6.** Energy-resolved spectra of 5α-androstane-3,17β-diols **3** and **4**. The analytes were dissolved in methanol–water (50:50) and introduced using a micro-heated nebulizer interface with positive ionization and 'up-front' CID. Data were acquired in the SIM mode.

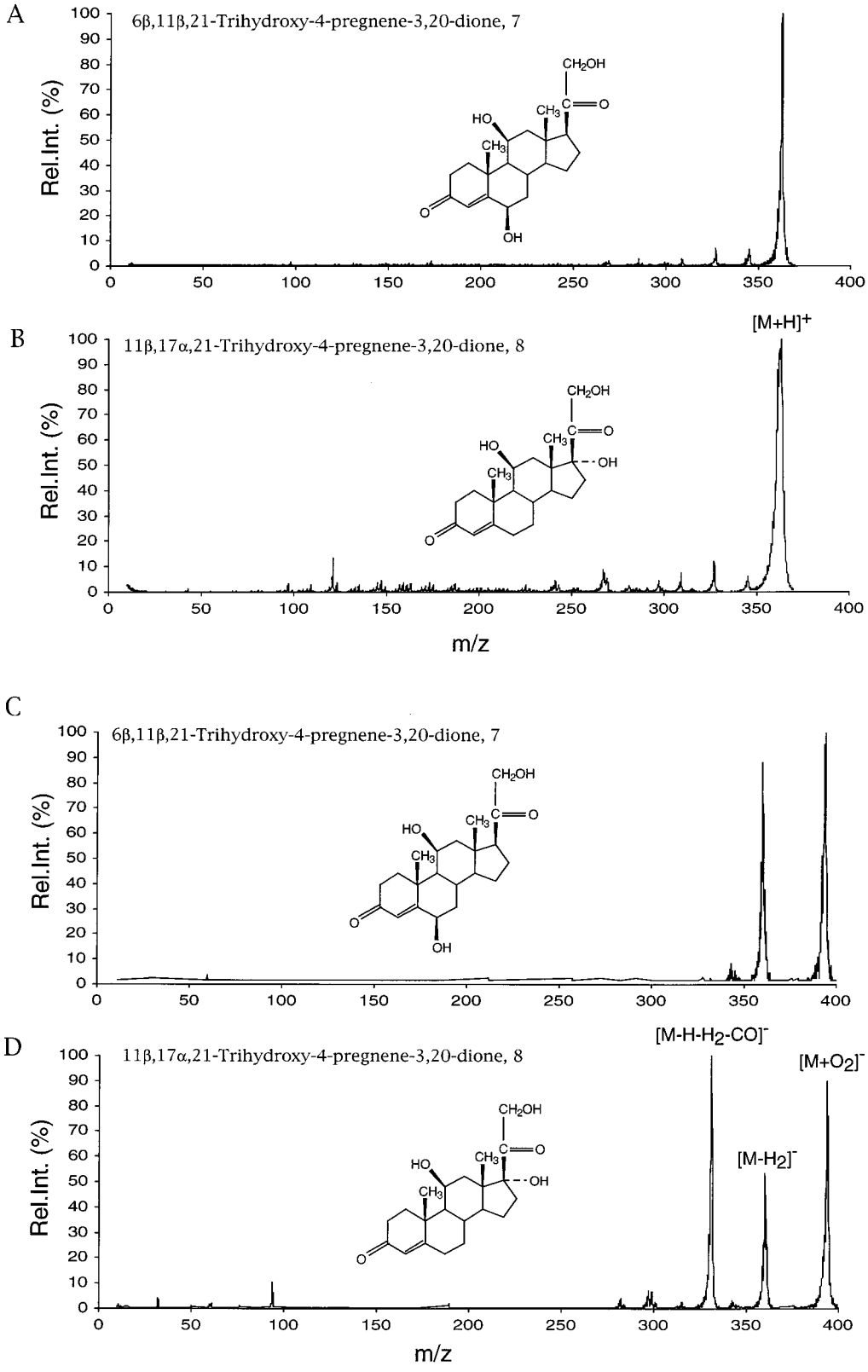



**Figure 7.** Tandem mass spectra of 6$\beta$,11$\beta$,21-trihydroxy-4-pregnene-3,20-dione (**7**) and 11$\beta$,17$\alpha$,21-trihydroxy-4-pregnene-3,20-dione (**8**) with (a,b) positive ionization and (c,d) negative ionization. The analytes were introduced using SFE with the collision gas thickness was adjusted to $200 \times 10^{12}$ molecules $cm^{-2}$ with a collision energy of 50 eV.

## REFERENCES


1. K. Nakanishi, T. Goto, S. Itô, S. Natori and S. Nozoe (Eds), *Natural Products Chemistry*, Chapt. 6. Kodansha/Academic Press, Tokyo (1974).
2. Z. V. I. Zaretskii, in *Mass Spectrometry of Steroids*. Wiley, New York (1976).
3. J.-C. Tabet, in *Methods in Stereochemical analysis: Application of Mass Spectrometry to Organic Stereochemistry*, edited by J. S. Splitter and F. Turecek, Chapt. 19. VCH, New York (1994).
4. J. H. Beynon, R. G. Cooks, J. W. Amy, W. E. Baitinger and T. Y. Ridley, *Anal. Chem.* **45**, 1023A (1973).
5. A. G. Harrison, in *Chemical Ionization Mass Spectrometry*, 2nd edn. CRC, Boca Raton, FL (1992).
6. J. G. Liehr, E. E. Kingston and J. H. Beynon, *Biol. Mass Spectrom.* **12**, 95 (1985).
7. E. C. Huang, T. Wachs, J. J. Conboy and J. D. Henion, *Anal. Chem.* **62**, 713A (1990).
8. A. P. Bruins, *Mass Spectrom. Rev.* **10**, 53 (1991).
9. A. P. Bruins, *Trends Anal. Chem.* **13**, 37 (1994).
10. P. H. Dawson and D. J. Douglas, in *Tandem Mass Spectrometry*, edited by F. W. McLafferty, Chapt. 6. Wiley, New York (1983).
11. R. A. Yost and C. G. Enke, in *Tandem Mass Spectrometry*, edited by F. W. McLafferty, Chapt. 8. Wiley, New York (1983).
12. V. H. Wysocki, in *Mass Spectrometry in the Biological Sciences: a Tuturial*, edited by M. L. Gross, pp. 59–77. Kluwer, Dordrecht (1992).
13. J. Ding, W. Burkhart and D. B. Kassel, *Rapid Commun. Mass Spectrom.* **8**, 94 (1994).
14. R. D. Smith, J. A. Loo, C. J. Barinaga, C. G. Edmonds and H. R. Udseth, *J. Am. Soc. Mass Spectrom.* **1**, 53 (1990).
15. M. J. Huddleston, M. F. Bean and S. A. Carr, *Anal. Chem.* **65**, 877 (1993).
16. R. D. Voyksner and T. Pack, *Rapid Commun. Mass Spectrom.* **5**, 263 (1991).
17. P. H. Dawson, J. B. French, J. A. Buckley, D. J. Douglas and D. Simmons, *Org. Mass Spectrom.* **17**, 205 (1982).
18. E. C. Horning, M. G. Horning, D. I. Carroll, I. Dzidic and R. N. Stillwell, *Anal. Chem.* **45**, 936 (1973).
19. J. B. French, B. A. Thomson, W. R. Davidson, N. M. Reid and J. A. Buckley, in *Mass Spectrometry in Environmental Sciences*, edited by F. W. Karasek, O. Hutzinger and S. Safe, p. 101. Plenum Press, New York (1985).
20. M. W. Wensing, A. P. Snyder and C. S. Harden, *Rapid Commun. Mass Spectrom.* **10**, 1259 (1996).
21. H. Kambara, Y. Mitsui and I. Kanomata, *Anal. Chem.* **51**, 1447 (1979).
22. M. W. Wensing, A. P. Snyder and C. S. Harden, *J. Mass Spectrom.* **30**, 1539 (1995).
23. L. N. Tyrefors, R. X. Moulder and K. E. Markides, *Anal. Chem.* **65**, 2835 (1993).
24. P. J. R. Sjöberg and K. E. Markides, *J. Chromatogr. A* **785**, 101 (1997).
25. L. M. Nyholm, P. J. R. Sjöberg and K. E. Markides, *J. Chromatogr. A* **755**, 153 (1996).
26. R. A. Yost, C. G. Enke, D. C. McGilvery, D. Smith and J. D. Morrison, *Int. J. Mass Spectrom. Ion Phys.* **30**, 127 (1979).
27. I. Dzidic, D. I. Carroll, R. N. Stillwell and E. C. Horning, *Anal. Chem.* **47**, 1308 (1975).
28. I. Dzidic, D. I. Carroll, R. N. Stillwell and E. C. Horning, *J. Am. Chem. Soc.* **96**, 5258 (1974).
29. A. P. Bruins, *Int. J. Mass Spectrom. Ion Phys.* **48**, 185 (1983).
30. M. Morris, P. Thibault and R. K. Boyd, *J. Am. Soc. Mass Spectrom.* **5**, 1042 (1994).
31. B. A. Thomson, D. J. Douglas, J. J. Corr, J. W. Hager and C. L. Jolliffe, *Anal. Chem.* **67**, 1696 (1995).
32. J. Michnowicz and B. Munson, *Org. Mass Spectrom.* **6**, 765 (1972).
33. D. Promé, J. Promé and D. Stahl, *Org. Mass Spectrom.* **20**, 525 (1985).